The Role of the Mitochondrial Permeability Transition Pore in Myocardial Protection

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ABSTRACT

Background- With coronary artery disease set to become the world's leading cause of mortality by 2020, there is an urgent requirement for novel treatment strategies which protect the myocardium against ischaemia-reperfusion injury, in order that patient morbidity and mortality can be improved and the global burden of this disease can be alleviated. This thesis examined the role of the mitochondrial permeability transition pore (mPTP) as a novel target for two major strategies for myocardial protection: (1) Interventions applied at the time of reperfusion, which protect the heart against lethal reperfusion injury, and (2) Myocardial Preconditioning, a process which renders the myocardium more resistant to subsequent ischaemia-reperfusion injury.

Methods and Results- Using an isolated perfused rat model of ischaemia-reperfusion injury, we demonstrated that the opening of the mPTP at the time of reperfusion is a critical determinant of myocyte death, and we have shown that inhibiting its opening, by administering pharmacological agents at the time of reperfusion, is cardio-protective. Using experimental models for inducing and detecting mPTP opening in adult rat mitochondria and myocytes, we demonstrated that myocardial preconditioning protects the heart by inhibiting mPTP opening, and that the pro-survival kinases Akt and Erk1/2, may act to mediate the preconditioning-induced inhibition of mPTP opening at the time of reperfusion. Finally, we found that the mPTP may also act as a mediator of the preconditioning signal. In this scenario, transient (low-conductance) opening of the mPTP, which does not lead to cell death, may paradoxically contributed to protective mechanisms recruited by myocardial preconditioning and mitochondrial-uncoupling, indicating a dual role for the mPTP in myocardial preconditioning.

Conclusion- We have demonstrated that inhibiting the opening of the mitochondrial permeability transition pore at the time of reperfusion, presents a common target for myocardial protection, irrespective of whether protection is mediated by myocardial preconditioning or by interventions applied solely at the time of reperfusion. Therefore, interventions which target and inhibit mPTP opening, at the time of reperfusion, may improve morbidity and mortality from coronary artery disease, in the clinical settings of ischaemia-reperfusion injury such as thrombolysis following an acute myocardial infarction, heart surgery and percutaneous transluminal coronary angioplasty. Furthermore, we found that transient (low-conductance) opening of the mPTP mediates both preconditioning and mitochondrial uncoupling-induced protection by acting as a channel for the mitochondrial release of reactive oxygen species.

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LIST OF ABBREVIATIONS

The following is a list of abbreviations used in this thesis.

$\Delta\psi_m$	mitochondrial membrane potential
%	percentage
ABC	ATP-binding cassette protein
ACE-I	angiotensin-converting enzyme inhibitor
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
Akt	cellular Akt/ protein kinase B
AMISTAD	Acute Myocardial Infarction STudy of ADenosine
AMP	adenosine monophosphate
ANOVA	analysis of variance
ANT	adenine nucleotide translocase
APAF-1	apoptosis protease-inducing factor-1
APD	action potential duration
AP-1	activating protein-1
AR	aldose reductase
ATP	adenosine triphosphate
ATPase	ATP synthase
B2	bradykinin B2 receptor
Ba^{2+}	barium
Bad	Bcl-X_L/Bcl-2-associated death promoter
Bax/BAX	Bcl-associated X protein
BCA	bicinchoninic acid
BCl_2	B-cell lymphoma 2 gene
BMK	big MAP kinase
^{14}C	radioactive carbon
Ca^{2+}	calcium ion
Caspase	cystein aspartate specific proteases
CCPA	2-chloro N^6 cyclopentyl adenosine

CFR	coronary flow rate
cGMP	cyclic guanine-5-monophosphate
CK	creatine kinase
CPC	calcium preconditioning
CsA	cyclosporin-A
CT-1	cardiotrophin-1
Cx	connexin
Da	Dalton
DCF	dichlorofluorescin
DIABLO	direct IAP-binding protein with low pI
DMSO	dimethyl sulphoxide
EDTA	ethylene diamine tetracetic acid
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol-tetra acetate
eNOS	endothelial nitric oxide synthase
Endo G	endonuclease G
Erk	extracellular signal-regulated MAPK
ESCAMI	Evaluation of the Safety and Cardio-protective Effects of Eniporide in Acute Myocardial Infarction
ETC	electron transport chain
F_0F_1-ATPase	ATP synthase
$FADH_2$	flavin adenine dinucleotide
FGF	fibroblast growth factor
5-HD	5-hydroxydecanoic acid
FK506	Tacrolimus
GST	glutathione-*S*-transferase
GUARDIAN	Guard During Ischaemia Against Necrosis
HK	hexokinase
HMG-CoA	hydroxyl-3-methylglutaryl-co-enzyme A
H_2O_2	hydrogen peroxide
HSP	heat shock protein
gp130	glycoprotein 130

GPCR	G-protein coupled receptor
H^+	hydrogen ion/proton
HtrA2	high temperature requirement A2
IC_{50}	50% inhibitory concentration
IGF-1	insulin-like growth factor 1
IMAC	inner membrane anion channel
IMM	inner mitochondrial membrane
iNOS	inducible nitric oxide synthase
IONA	Impact Of Nicorandil on Angina
IPC	ischaemic preconditioning
I/R%	infarct-risk volume ratio
ISIS-IV	the fourth International Study of Infarct Survival
JAK	Janus kinase
JNK	c-Jun NHP_2 terminal kinase
K^+	potassium ion
K_{ATP}	ATP-sensitive potassium channel
kDa	kilodalton
K_i	dissociation constant for inhibitor binding
Kir	inwardly-rectifying K^+ channel
LDH	lactate dehydrogenase
LAD	left anterior descending
LIMIT-2	Second Leicester Intravenous Magnesium Intervention Trial
L-NAME	N ω-nitro-L-arginine methyl ester
M-ABC	mitochondrial ATP binding cassette protein
Mg^{2+}	magnesium ion
MAPK	mitogen activated protein kinase
MAPKAPK	MAPK- activated protein kinase
MAPKK/MKK	MAPK- activated protein kinase
MAPKKK/MKKK	MAPK- activated protein kinase kinase
MEF	myocyte enhancing factor
MEKK	MAPK/Erk kinase
MBR	mitochondrial benzodiazepine receptor

mK_{ATP}	mitochondrial K_{ATP}
MMC	mitochondrial megachannel
MMP	mitochondrial membrane permeabilisation
Mn^{2+}	manganese
MnSOD	manganese superoxide dismutase
mPTP	mitochondrial permeability transition pore
Na^+	sodium ion
NADH	nicotinamide adenine dinucleotide
nm	nanometres
NF-κB	nuclear factor kappa B
NHE	Na^+-H^+ exchanger
NO	nitric oxide
$\cdot O_{2-}$	superoxide anion
·OH	hydroxyl radical
OMM	outer mitochondrial membrane
PBS	phosphate buffered saline
PDK	3-phosphoinositide-dependent protein kinase
PG	prostaglandin
pH	pH
P_i	inorganic phosphate
PI3K	phosphatidyl inositol 3-OH kinase
PKC	protein kinase C
PKG	protein kinase G
PLSD	protected least significance difference
PPIase	peptidyl prolyl transisomerase
p70S6K	70-kDA ribosomal protein S6 kinase
p90RSK	p90 ribosomal S6 kinase
RACK	receptors of activated C kinase
Raf	MAPK kinase
RISK	reperfusion injury salvage kinase
ROS	reactive oxygen species
RPP	rate pressure product

RTK	receptor tyrosine kinase
SDS	sodium dodecylsuphate
SfA	sanglifehrin-A
SMAC	second mitochondrial activator of caspases
SNAP	S-nitro N-acetyl penicillamine
SPT	8-p-sulphonyphenyl theophylline
Sr^{2+}	strontium
Src	sarcoma Rous virus
STAT	signal transducer and activator of transcription
SDH	succinate dehydrogenase
SUR	sulphonylurea receptor
SWOP	second window of protection
3-NPA	3-nitropropionic acid
TGF-β1	transforming growth factor-β1
TK	tyrosine kinase
TMRM	tetramethyl-rhodamine methyl ester
2-DOG	2-deoxyglucose
VDAC	voltage-dependent anion channel

LIST OF PUBLICATIONS

The following is a list of publications arising from the thesis.

Original Articles

Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM. Inhibiting mitochondrial permeability transition pore: a new paradigm in myocardial preconditioning? *Cardiovasc Res* 2002:55;534-543.

Hausenloy D J, Duchen MR, Yellon, DM. Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia-reperfusion injury. *Cardiovasc Res* 2003: 60;617-625.

Hausenloy DJ, Wynne A, Duchen, MR, Yellon DM. Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. *Circulation* 2004:109;1714-1717.

Hausenloy DJ, Yellon DM, Mani-Babu S, Duchen MR. Preconditioning protects myocytes against oxidative stress by inhibiting mitochondrial permeability transition pore opening. *Am J Physiol* 2004:Apr 8 [Epub ahead of print].

Hausenloy DJ, Mocanu, M, Yellon DM. Cross-talk between the survival kinases during early reperfusion: its contribution to ischemic preconditioning. *Cardiovasc Res* 2004:In-Press.

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Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Risk Kinase (RISK)-pathway. *Cardiovasc Res* 2004:61;448-460.

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Hausenloy DJ, Yellon DM. The mitochondrial permeability transition pore: its fundamental role in mediating cell death during ischaemia and reperfusion. *J Mol Cell Cardiol* 2003:35;339-341.

Letters

Hausenloy DJ, Yellon DM. Adenosine-induced second window of protection is mediated by inhibition of mitochondrial permeability transition pore opening at the time of reperfusion. *Cardiovasc Drugs Therapeutics* 2004:In-Press.

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Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM. Inhibiting mitochondrial permeability transition pore opening: a new paradigm in myocardial preconditioning? *Brit J of Pharm* 2001;135:323.

Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM. Inhibiting mitochondrial permeability transition pore opening:a new paradigm in myocardial preconditioning? *J Mol Cell Cardiol* 2002: 34(6); A28.

Hausenloy DJ, Mani-Babu S, Duchen MR, Yellon DM. Mitochondrial K_{ATP} channel activation protects the myocyte against oxidative stress by inhibiting mitochondrial permeability transition pore opening. *Circulation* 2002;106:I-1160.

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Hausenloy DJ, Mani-Babu S, Duchen MR, Yellon DM. Preconditioning via mitochondrial K_{ATP} channel activation protects cardiomyocytes from oxidative stress by inhibiting mitochondrial permeability transition pore opening. *Medical Research Society* 2003:M18

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Hausenloy DJ, Mocanu M, Yellon DM. Activation of the pro-survival kinase cascades (PI3 Kinase-Akt-p70S6K and Erk 1/2-p70S6K) at reperfusion are essential for preconditioning-induced protection. *Circulation* 2003;108:I-288.

Hausenloy DJ, Mocanu, M, Yellon DM. Cross-talk between the survival kinases during early reperfusion: its contribution to ischemic preconditioning. To be presented at ISHR 2004.

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Chapter One: GENERAL INTRODUCTION

1.1 Coronary Artery Disease

Coronary artery disease and its many sequelae is currently the leading cause of mortality and morbidity in the Western World, and by the year 2020, it is poised to become the leading cause of mortality in the World.([1]) Therefore, the magnitude of the problem and the global burden it imposes now and in the immediate future, necessitates a concerted research effort both in the areas of primary prevention and secondary prevention of coronary artery disease. Ischaemic heart disease encompasses a continuum of disease states ranging from sub-clinical coronary artery disease to chronic stable angina, through to unstable angina, right up to an acute myocardial infarction, and its incumbent consequences such as congestive cardiac failure. Targeting the different stages of this disease process remains a common goal for researchers based in the basic laboratory as well as those in the clinical environment. Importantly, it is the translation of basic research findings made in the laboratory setting (the so-called 'bench to the bedside' approach), which will make the largest impact on tackling the consequences of coronary artery disease, in the clinical arena.

The serious and often fatal consequence of coronary artery disease culminate from the rupture of an unstable atherosclerotic plaque, which results in the formation of a thrombotic plug which occludes one of the major coronary arteries, and presents clinically as an acute myocardial infarction.([2;3]) In this scenario, the best hope of salvaging myocardium for the two thirds of patients presenting with an acute myocardial infarction that make it alive to the hospital,([4]) is the timely restoration of coronary blood flow to the ischaemic myocardium, by either thrombolysis or primary percutaneous transluminal coronary angioplasty (PTCA). By limiting the size of an evolving myocardial infarct, through the employment of these reperfusion strategies, the ultimate goal is to maintain and optimise left ventricular function, since it is the residual left ventricular function, which determines the patient's prognosis.

Therefore, in order to realise an improvement in the mortality and morbidity of this disease, it is necessary to discover novel treatment strategies which provide protection to the myocardium from ischaemia-reperfusion injury. Treatment strategies which protect the myocardium from the myocyte injury that results from an acute coronary artery occlusion, can be broadly classified into two categories:

(1) Treatment strategies that intervene ***before*** the index ischaemic event: these are difficult to apply in the clinical arena, given the unpredictable onset of an acute coronary artery occlusion.

(2) Treatment strategies which intervene ***after*** the onset of ischaemia but before or at the time of therapeutic reperfusion: these protect the heart against lethal reperfusion injury, and are easier to apply, as the onset of reperfusion is under the control of the operator. In this scenario the intervention can be administered as an adjunct to current reperfusion therapy.

One of the most powerful mechanisms for protecting the myocardium ***before*** the acute coronary artery occlusion occurs, is to ischaemically precondition the myocardium, a phenomenon which was first described in the seminal study by Murry and colleagues in 1986.([5]) This manoeuvre renders the myocardium more resistant to the ensuing lethal ischaemic period, by inducing an innate cellular adaptation response to stress. This approach, however depends crucially on intervening ***before*** the ischaemic event, which is difficult, given the unpredictable timing of an acute coronary artery occlusion.

The restoration of coronary blood flow, following an acute coronary artery occlusion is essential for myocardial salvage, but paradoxically, it is also accompanied by injury to the myocardium, termed lethal reperfusion injury.([6]) Therefore, protecting the myocardium against the lethal reperfusion injury, which results from opening the occluded coronary artery, offers an alternative clinically amenable target for cardio-protection, given that the onset of reperfusion is predictable, and is under the control of the operator.

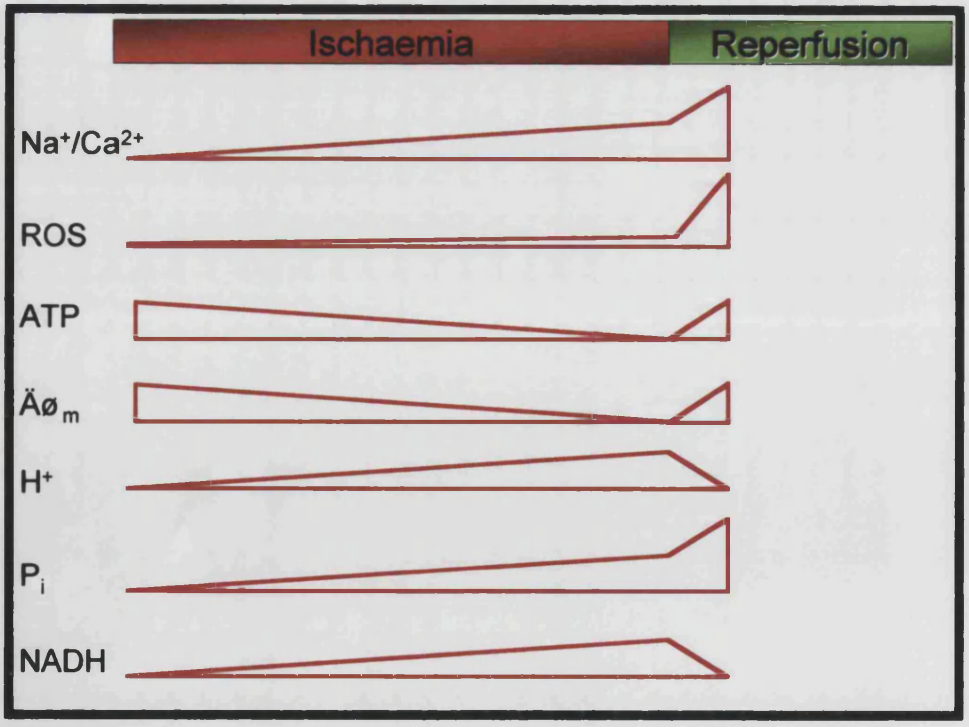
This thesis explores both these mechanisms of protecting the myocardium from ischaemia-reperfusion injury. Despite being separated in terms of the timing of the intervention and the conceived mechanisms of action, these cardio-protective strategies may both converge on the main subject of this thesis, the mitochondrial permeability transition pore (mPTP), which was first described in the late 1970's by the pioneering studies of Haworth and Hunter.([7-10]) The mPTP is emerging as a key player in the arena of myocardial protection, and it is the aim of this thesis to explore its critical role in ischaemia-reperfusion injury, and to examine its contribution to the setting of ischaemic preconditioning.

An acute myocardial infarction is accompanied by abrupt biochemical and metabolic changes, which occur during the ischaemic period as well as at the time of reperfusion, when the occluded coronary artery is opened. An appreciation of the biochemical changes which take place during ischaemia-reperfusion injury provides the context for understanding the mechanisms involved in the cardio-protective strategies explored in this thesis.

1.2 Myocardial Ischaemia-Reperfusion Injury

An acute coronary artery occlusion results in a critical reduction in coronary blood flow and deprivation of oxygen and nutrients to the affected area of myocardium, and reduces the clearance of waste metabolites, subjecting myocytes to the abrupt metabolic and biochemical changes associated with myocardial ischaemia. If blood flow is restored to the affected area by the removal of the coronary artery occlusion, the ischaemic myocytes are then exposed to the further metabolic and biochemical changes associated with the reperfusion process. The combined injury sustained by the myocardium during these processes is termed myocardial ischaemia-reperfusion injury, and the sequential metabolic and biochemical perturbations that occur during this process are reviewed in this section and presented diagrammatically in figure 1.1.

Figure 1.1: The Metabolic and Biochemical Changes Which Occur During Ischaemia and Reperfusion. During myocardial ischaemia there is an increase in intracellular Ca^{2+}, P_i, Na^+, reactive oxygen species (ROS), NADH, H^+ and a fall in both ATP levels and the mitochondrial membrane potential ($\Delta\psi_m$). At reperfusion there is repolarisation of the $\Delta\psi_m$ and restoration of ATP production, a further increase in Ca^{2+}, P_i, ROS, oxidation of NADH and restoration of physiological pH.



1.2.1 Metabolic and Biochemical Consequences of Myocardial Ischaemia

Deprivation of oxygen during ischaemia impairs oxidative phosphorylation by reducing electron flow through the electron transport chain, leading to an accumulation of NADH and FADH and cessation of ATP production.([11]) The mitochondrial membrane potential collapses as it is no longer maintained by the electrochemical gradient maintained across the inner mitochondrial membrane.([12]) Intracellular creatine phosphate is depleted with a concomitant rise in intracellular P_i, resulting in mitochondria accumulating P_i.([13]) Residual reserves of ATP are hydrolysed by F_0F_1-ATPase in an attempt to restore the mitochondrial membrane potential,([14;15]) resulting in catalytic metabolites such as hypoxanthine which are oxidised to release free radicals. The activity of the adenine nucleotide translocase is reduced impairing oxidative phosphorylation still further.([16])

The reduced availability of ATP and oxygen drives anaerobic glycolysis which results in lactic acid accumulation, leading to a decrease in intracellular pH.([11]) The fall in pH activates the Na^+/H^+ exchanger in an effort to remove cytosolic protons, which causes the entry of Na^+. The Na^+/K^+-ATPase which normally removes excess Na^+ is inhibited because of the reduced availability of ATP and the increase in intracellular phosphate. This causes a rise in intracellular Na^+ which triggers the Na^+/Ca^{2+} exchanger to function in reverse, in order to remove cytosolic Na^+ ([17])- however, this occurs at the expense of an increase in intracellular Ca^{2+}. The rise in cytosolic Ca^{2+} results in the mitochondrial accumulation of Ca^{2+} via the mitochondrial Na^+/Ca^{2+} exchanger.([18]) The onset of rigour contracture, which times with the depletion of ATP,([19]) is followed by cellular Ca^{2+} overload.([20])

Therefore, following an episode of sustained myocardial ischaemia, the metabolic and biochemical derangements include: a low intracellular pH (<7.0), high intracellular [Ca^{2+}] and [P_i], and ATP depletion (see figure 1.1). However, these conditions are drastically changed once the ischaemic myocardium is reperfused.

1.2.2 Metabolic and Biochemical Consequences of Myocardial Reperfusion

Reperfusion of an ischaemic myocardium has several important consequences: (1) re-energisation of the myocyte which causes repolarisation of the mitochondrial membrane potential; (2) reoxygenation of a reduced mitochondrial respiratory chain resulting in the

production of reactive oxygen species and oxidation of NADH/FADH (3) a drop in intracellular Ca^{2+} but a further influx of Ca^{2+} into mitochondria via the Ca^{2+}-uniporter driven by the recovered mitochondrial membrane potential;([20-22]) (4) the wash-out of lactic acid, which in combination with the reactivation of the Na^{+}/H^{+} exchanger, acts to restore a physiological pH. The changes which occur at the time of reperfusion are discussed in more detail in section 1.4.1 in the context of lethal reperfusion injury. Many of the biochemical and metabolic changes which take place during the first few minutes of reperfusion can mediate cell death by inducing the opening of the mitochondrial permeability transition pore (mPTP), which is discussed later in section 1.5.

Therefore, the ultimate aim of any cardio-protective strategy which is designed to protect the myocyte against the combined effect of ischaemic and reperfusion injury, is to preserve cellular energetic and ionic homeostasis during this insult. In this regard, by rendering the myocardium more resistant to the effects of ischaemia-reperfusion injury, ischaemic preconditioning presents a powerful cardio-protective strategy and is reviewed in the following section.

1.3 Myocardial Protection by Ischaemic Preconditioning

Following the discovery in 1984, that repeated brief episodes of myocardial ischaemia did not produce cumulative deficits in either adenine nucleotide content([23]) or contractile function,([24]) Reimer and colleagues went on to demonstrate that four 10 minute episodes of myocardial ischaemia did not induce myocardial necrosis or cause a cumulative loss in ATP, when compared to a sustained 40 minute period of ischaemia.([25]) Based on these findings, Reimer and colleagues embarked on a study, the findings of which would go on to make a major impact in the field of myocardial protection.([5]) In what has become a seminal study, these investigators demonstrated, using a canine model of ischaemia-reperfusion injury, that four 5 minute alternate episodes of circumflex coronary artery occlusion and reperfusion, applied before a sustained 40 minute occlusion, reduced infarct size to 25% of that observed in the control group.([5]) The investigators termed this protection, ischaemic preconditioning (IPC), and suggested that this protective effect appeared to delay cell death, as the protective effect was lost if sustained ischaemia was extended to 3 hours.([5]) An alternative explanation may be, that timely reperfusion is still a pre-requisite for limiting infarct size. They also demonstrated that IPC resulted in a permanent reduction in infarct size, as the protection was still present after 4 days of reperfusion.([5])

The clinical potential of such a powerful protective phenomenon has generated enormous interest in identifying the underlying intracellular signalling pathways, and has resulted in the publication of nearly 4000 studies over the last 17 years, leading some commentators to describe ischaemic preconditioning as "the strongest form of in vivo protection against myocardial ischemia other than early reperfusion".([26]) Despite the magnitude of this research effort the exact mechanism through which ischaemic preconditioning protects the heart against a subsequent lethal ischaemic episode is not completely understood.

Characteristics of Ischaemic Preconditioning

The phenomenon of ischaemic preconditioning exerts robust and reproducible protection and appears to be a ubiquitous endogenous protective mechanism that has been observed: (a) in the heart of every species tested, including rat,([27]) mice,([28]) pig,([29]) rabbit,([30]) ferret,([31]) guinea pig,([32]) sheep([33]) and man([34]); (b) at the cellular level (reviewed in reference ([35])); (c) at the

mitochondrial level;([36]) (d) in other organs such as liver,([37]) kidney,([38]) gut,([39]) skeletal tissue,([40]) urinary bladder([41]) and brain.([42])

The cardio-protective effect of ischaemic preconditioning disappears if the time interval between the preconditioning ischaemic episode and the subsequent sustained lethal ischaemic episode is extended beyond 3-4 hours,([43]) suggesting that the protection induced by ischaemic preconditioning is transient. In rat hearts, 5 minutes of ischaemia is required to induce the preconditioning effect, whereas in the rabbit and canine hearts, 2 minutes of ischaemia may be sufficient to elicit a preconditioning-induced reduction in infarct size.([43]) This early transient form of ischaemic preconditioning-induced protection has been termed *classical* preconditioning.

In 1993, two laboratories independently described the reappearance of preconditioning-induced protection in terms of a reduction in infarct size, 24 hours after the onset of the preconditioning stimulus, in rabbit([44]) and canine([45]) myocardium, a phenomenon that has been termed the second window of protection (SWOP) or delayed preconditioning.([44])

Indicators of IPC-Induced Protection

The original end-point used to assess the protection induced by ischaemic preconditioning was the reduction in infarct size.([5]) However, ischaemic preconditioning has also been demonstrated to protect against ventricular arrhythmias,([46]) and improve the recovery of post-ischaemic contractile function in isolated hearts.([47]) Whether IPC protects against the phenomenon of myocardial stunning, which describes the impairment of left ventricular contractile function induced by a sub-lethal episode of ischaemia (usually less than 15 minutes), is unresolved (reviewed in reference ([48])).

To aid the identification of the intracellular pathways implicated in ischaemic preconditioning, investigators have developed various models which simulate the phenomenon of ischaemic preconditioning at the tissue and cellular level. In these models ischaemia is simulated by: (a) subjecting the cells to hypoxia (which is achieved by either using an hypoxic chamber([49]) or by centrifuging the cells into an ischaemic pellet and covering the cells with a layer of mineral oil([50;51])), or (b) by subjecting the cells to metabolic inhibition.([52]) By applying a brief episode of the simulated ischaemia prior to the period of simulated lethal ischaemia, these investigators have demonstrated 'preconditioning-like' protection, in terms of enhanced viability, assessed either morphologically or by measuring the reduction in staining with either propidium iodide or trypan blue (markers of necrotic cell death). Preconditioning has also been

demonstrated in isolated rabbit ventricular trabeculae, in which a brief exposure of simulated ischaemia, reproduced by superfusing with hypoxic buffer, can protect the tissue against a sustained hypoxic period, as evidence by an improved recovery of contractile function.([53])

1.3.1 Classical Preconditioning

Despite on-going intensive investigation, the actual mechanism of protection associated with IPC remains unknown. However, the components which make up the signal transduction pathway involved in protection have been thoroughly explored, and will be reviewed first, followed by the possible mechanisms of protection that have been postulated to mediate IPC.

The signal transduction pathway can be conceptually classified into triggers (these are factors which act before the index ischaemic episode and activate downstream signalling mechanisms, and convey the 'memory' effect of preconditioning) or mediators/end-effectors (these are factors which act during or after the index ischaemic episode and mediate the protective effect). This separation is not rigid as certain components have been demonstrated to act as both triggers and mediators/effectors.

1.3.2 Triggers of Classical Preconditioning

1.3.2.1 G-Protein Coupled Receptor Ligands

In 1991, Downey's group examined the role of adenosine, which is generated during myocardial ischaemia from the hydrolysis of ATP, as a potential trigger of preconditioning.([30]) They demonstrated that the protection associated with IPC could be abolished by the pre-treatment of an adenosine antagonist, and that a 5 minute intra-coronary infusion of adenosine followed by 10 minutes of wash-out, could reproduce IPC-induced protection.([30]) The important implications from these findings included: (1) IPC-induced protection could be mimicked by pharmacological agents, with these agents acting as triggers of preconditioning (so-called pharmacological preconditioning); and (2) the endogenous activation of a G-protein coupled receptor (GPCR), was an essential component of IPC-induced protection , that is IPC was a receptor-mediated phenomenon.

Several studies followed, providing support for the involvement of GPCR activation as an essential trigger of preconditioning-induced protection, using other ligands, such as

bradykinin,([54;55]) opioids,([56]) acetylcholine,([57]) catecholamines,([58]) angiotensin II,([59]) and endothelin-1,([60]) some of which are released by the heart during ischaemia.

Interestingly, Goto and colleagues([55]) demonstrated that, pharmacologically blocking the bradykinin receptor could abrogate protection from a single cycle of preconditioning but not from multiple cycles, suggesting that ischaemic preconditioning via the GPCR, was a highly redundant phenomenon. Therefore, the blockade of a single receptor type served only to raise the ischaemic threshold required to trigger protection, rather than completely blocking it.([55]) The simultaneous activation of these GPCR's during brief ischaemia-reperfusion suggested that the preconditioning signal converged on a downstream target. Protein kinase C (PKC) was proposed to be the downstream target, based on the finding that: (a) PKC was phosphorylated by the activation of the GPCR's; ([58;59]) and (b) that the protection associated with bradykinin,([55]) adenosine,([61]) and opioids,([62]) could all be blocked by PKC inhibitors. The role of PKC as a mediator of preconditioning is reviewed later in section 1.3.3.1. PKC has also been demonstrated to be a downstream target of reactive oxygen species (ROS),([63]) a non-receptor trigger of preconditioning which is reviewed in the next section.

1.3.2.2 Reactive Oxygen Species

Reactive oxygen species (ROS), when present in low concentrations, in contrast to higher concentrations which are pro-injurious, can modify cellular activities and participate in cell signalling (reviewed in reference ([64])). In 1988, Murry and colleagues([65]) first demonstrated that antioxidants could abolish the preconditioning effect, implicating for the first time a role for ROS as a trigger of preconditioning, a finding which was later supported by several studies,([63;66;67]) but not all.([68-70]) The disparity in findings between these studies has been attributed to the type of antioxidant and the number of preconditioning cycles used in the different studies.([63])

Tritto and colleagues([71]) were the first to demonstrate directly, that a low dose of ROS could act as a preconditioning trigger and mimic IPC-induced protection. Subsequently, Vanden Hoek and colleagues([49]) demonstrated, using chick neonatal myocytes, that hypoxic preconditioning produced a burst of ROS (mainly H_2O_2), as measured by dichlorofluorescin fluorescence, from complex III of the mitochondrial electron transport chain, which appeared to exit mitochondria via the inner membrane anion channel (IMAC). The mechanism by which preconditioning results in the mitochondrial generation of ROS is unclear but recent studies

implicate the involvement of the mitochondrial ATP-sensitive K^+ (K_{ATP}) channel (see section 1.3.2.4).([72-74])

Once released into the cytosol, mitochondrial-derived ROS are believed to activate protein kinases such as PKC,([63]) Erk1/2,([75]) p38 MAPK,([76]) which then act as mediators of the preconditioning signal (see section 1.3.3). The modulation of ROS production at the time of reperfusion in IPC also implicates ROS as a potential end-effector of IPC-induced protection, in addition to its proposed role as a trigger of preconditioning (see section 1.3.4.4).([77-79])

The actual intracellular pathway through which an IPC stimulus received at the cell membrane results in the mitochondrial release of ROS is also unclear, although the activation of the phosphatidyl inositol 3-OH kinase (PI3K)-Akt cascade has been implicated.([80])

1.3.2.3 The PI3K-Akt Kinase Cascade

Recent studies have implicated signalling through the pro-survival, phosphatidyl inositol 3-OH kinase (PI3K)-Akt cascade, during the preconditioning phase before the index ischaemic episode, in IPC-induced protection. Tong and colleagues([81]) were the first to demonstrate that IPC activates the PI3K-Akt kinase cascades prior to the index ischaemic episode, and they showed that inhibiting Akt activity, using the PI3K inhibitor, wortmannin, abolished IPC-induced protection, using the recovery of function as the end-point. Yellon's group([82]) confirmed these findings in the isolated perfused rat heart infarct model. Interestingly, an earlier study by Baines and colleagues,([83]) had demonstrated that insulin-induced preconditioning also required the activation of the PI3K-Akt kinase cascade prior to the index ischaemic episode.

Activation of the GPCR by the preconditioning mimetic, acetylcholine, has been demonstrated to activate the PI3K-Akt kinase cascade prior to the index ischaemic period, suggesting that the GPCR may be the upstream activator of the PI3K-Akt kinase cascade, in the setting of IPC.([74;80;84]) Activation of the PI3K-Akt kinase was demonstrated to occur via the activation of Src tyrosine kinase and the epidermal growth factor receptor.([85])

Tong and colleagues([86]) have demonstrated that signalling through the PI3K-Akt kinase cascade results in the phosphorylation and inactivation of glycogen synthase kinase 3β, an enzyme originally identified for its role in regulating glycogen synthesis in response to insulin.([87;88]) Inactivation of this enzyme has been demonstrated to reduce apoptosis and enhance cell survival.([89]) The anti-apoptotic effects of the PI3K-Akt kinase cascades are

described in more detail in the context of protecting the heart against lethal reperfusion injury (see section 1.4.3.2).

Downey's laboratory([74;80]) have implicated the PI3K-Akt signalling cascade as a trigger of preconditioning, such that it relays the preconditioning signal from the GPCR at the cell membrane to the mitochondrial K_{ATP} channel, where the opening of the latter mediates the mitochondrial release of ROS. The intracellular signalling pathway through which the activation of the PI3K-Akt kinase cascade results in the opening of the mitochondrial K_{ATP} channel is not clear although nitric oxide has been implicated (see section 1.3.2.4.b).([90]) Interestingly, insulin-mediated recruitment of the PI3K-Akt did not appear to signal to the mitochondrial K_{ATP} channel or PKC, suggesting an alternative pathway of protection in this setting.([83])

Many of these trigger mechanisms converge on the mitochondria, and they appear to target the mitochondrial K_{ATP} channel in particular, which is reviewed in the next section.

1.3.2.4 The K_{ATP} Channel and Cardio-Protection

The role of the K_{ATP} channel in ischaemic preconditioning-induced protection has had an interesting and often controversial history. In 1983, Noma first identified an ATP-sensitive potassium (K_{ATP}) channel in isolated guinea pig ventricular myocytes.([91]) In this landmark discovery, Noma hypothesised that the opening of this channel in response to hypoxia or ischaemia, would protect the heart by enhancing the shortening of the action potential duration (APD), thereby providing a repolarising K^+ current, the effect of which would be to reduce cellular Ca^{2+} loading during ischaemia-reperfusion.([91]) Cole and colleagues([92]) provided the evidence for this proposition, by demonstrating that glibenclamide (a non-specific K_{ATP} channel blocker) attenuated the ADP shortening observed during ischaemia, and that pinacidil (a K_{ATP} channel opener) accelerated ADP shortening and improved myocardial recovery in function, following ischaemia-reperfusion.

The K_{ATP} channel has been shown to comprise an octomeric complex consisting of four Kir (an inwardly-rectifying K^+ channel) subunits and four SUR (sulphonylurea receptor) subunits.([93;94]) The Kir forms the K^+ conductance pore of the channel and the SUR confers the channel's sensitivity to ATP and sulphonylureas, and is a member of the ATP-binding cassette protein family (ABC family).([93;94]) Currently it is believed that Kir6.2 and SUR2 make up the sarcolemmal K_{ATP} channel.([93])

Gross and colleagues([95;96]) were the first to link the opening of the K_{ATP} channel with IPC-induced protection, from studies in which they demonstrated that IPC-induced protection could be abolished by the K_{ATP} channel blockers, glibenclamide or 5-hydroxydecanoic acid (5-HD). At this time, IPC-induced protection in this setting was attributed to APD shortening.([97-99]) However, from 1994, several studies appeared which suggested that the cardio-protection associated with K_{ATP} channel opening was independent of APD shortening,([100;101]) a finding which appeared to also apply to IPC-induced protection.([50]) This suggested that the sarcolemmal K_{ATP} channel may not be responsible for the cardio-protection associated with K_{ATP} channel openers and IPC, which resulted in the hunt for an intracellular site of protection, which culminated in the identification and characterisation of a K_{ATP} channel in the mitochondrial inner membrane, the source of much interest and controversy in the field of cardio-protection.

1.3.2.4.a The Mitochondrial K_{ATP} channel and Cardio-Protection

The existence of an ATP-sensitive potassium channel in the mitochondrial membrane was first suggested by Inoue and colleagues in 1991.([102]) In patch-clamp experiments of giant mitoplasts, fused from rat liver mitochondria, they demonstrated a K^+-selective channel sensitive to ATP, glibenclamide and 4-aminopyridine.([102]) Garlid's group then purified from the inner mitochondrial membranes of rat liver and bovine heart mitochondria, a fraction containing mitochondrial K_{ATP} channel activity, and found that this channel shared some pharmacological properties with the sarcolemmal K_{ATP} channel, while possessing a distinct profile.([103])

In 1997, Garlid's group first demonstrated a role for the mitochondrial K_{ATP} channel in cardio-protection.([104]) In that study, inner mitochondrial membrane proteins comprising the mitochondrial K_{ATP} channel were reconstituted into proteoliposomes and were demonstrated to be 2000 times more sensitive to diazoxide, than the sarcolemmal K_{ATP} channel, in terms of inducing a K^+ current.([104]) Furthermore, at concentrations (30-100 µmol/l) that did not activate the sarcolemmal K_{ATP} channel or shorten the APD, diazoxide was demonstrated to cardio-protect the isolated perfused rat heart subjected to ischaemia-reperfusion, as evidenced by an improvement in the recovery of left ventricular contractile function and attenuated lactate dehydrogenase (LDH) release.([104]) The effects of diazoxide were blocked by the non-specific K_{ATP} channel blocker, glibenclamide (with a K_i of 1-6 µmol/l) and 5-hydroxydecanoic acid (with a K_i of 45-85 µmol/l),([104]) which is believed to be a mitochondrial K_{ATP} channel specific blocker,([104-106]) although this has recently been disputed (see section 1.3.2.4.d).([107])

In the same year, Marban's group([108]) provided further evidence for the role of the mitochondrial K_{ATP} channel in cardio-protection, using rabbit ventricular myocytes, in which diazoxide (at 100 µmol/l) was demonstrated to: (1) induce a reversible increase in flavoprotein oxidation which was considered an indirect indicator of mitochondrial K_{ATP} channel opening, based on the premise that the opening of the mitochondrial K_{ATP} channel induces flavoprotein oxidation through mitochondrial uncoupling of oxidative phosphorylation. This effect of diazoxide was blocked by 5-HD using a concentration of 500 µmol/l but not at 100 µmol/l, which is in contrast to the study by Garlid's group, in which the K_i of 5HD was demonstrated to be 45-85 µmol/l;([104]) (2) protect the myocytes against simulated ischaemia-reperfusion injury; and (3) not induce opening of the sarcolemmal K_{ATP} channel.([108]) However, Standen's group([109]) who were able to demonstrate cardio-protection in rat ventricular myocytes subjected to simulated ischaemia-reperfusion, were unable to reproduce the flavoprotein oxidation and mitochondrial membrane depolarisation induced by diazoxide, as observed by Marban's group.([108]) It has now transpired, that to demonstrate the flavoprotein oxidation in response to diazoxide, the cells had been kept overnight in substrate-free medium, which would have de-energised them, allowing diazoxide to exert an uncoupling effect.([110]) The flavoprotein oxidation induced by diazoxide may also be due to in part to a non-specific effect of this drug on mitochondrial respiration, which occurs independently of the mitochondrial K_{ATP} channel, an issue which is discussed in section 1.3.2.4.d.

Because of the studies demonstrating that IPC-induced protection was sensitive to 5-HD, a presumed mitochondrial K_{ATP} channel blocker, and because of the studies demonstrating that opening of the mitochondrial K_{ATP} channel mediated cardio-protection,([104;108]) the next aim was to determine the role of the mitochondrial K_{ATP} channel in IPC-induced protection.

1.3.2.4.b The Mitochondrial K_{ATP} Channel as a Trigger of Preconditioning

The mitochondrial K_{ATP} channel was originally proposed to be the end-effector of IPC-induced protection,([104;108]) and this aspect is discussed in section 1.3.4.3, along with the possible mechanisms through which opening of the mitochondrial K_{ATP} channel is believed to mediate protection.

The role of the mitochondrial K_{ATP} channel as a trigger of preconditioning is a relatively recent development, first proposed by Downey's group.([72]) They and others([72;111]) have demonstrated that the transient opening of the mitochondrial K_{ATP} channel (for 5 minutes

followed by wash-out of the drug) could induce a preconditioned state for up to 30 minutes, after the application of the drug. They next demonstrated that both IPC and diazoxide-induced protection could only be abrogated if the K_{ATP} channel blockers were administered early to bracket the preconditioning stimulus.([72]) If the K_{ATP} channel blockers were given late, that is after the preconditioning stimulus but prior to the index ischaemic period, both IPC and diazoxide-induced protection was maintained, suggesting that the opening of the mitochondrial K_{ATP} channel was acting as a trigger of preconditioning, or in other words it was inducing the 'memory effect' associated with IPC.([72]) The role of the mitochondrial K_{ATP} channel as a preconditioning trigger has been questioned by several investigators who have proposed that the mitochondrial K_{ATP} channel may act as both a trigger and mediator/end-effector of preconditioning.([112-115])

The Relationship Between the Mitochondrial K_{ATP} Channel and ROS

Early studies had implicated ROS as a potential trigger of preconditioning ([65-67;71]) and complex III of the mitochondrial electron transport chain had been demonstrated to be the source of the ROS generated in response to a preconditioning stimulus (see section 1.3.2.2).([49;90]) Forbes and colleagues([116]) were the first to link mitochondrial ROS release with diazoxide-induced protection, thereby implicating the mitochondrial K_{ATP} channel in this scheme. They demonstrated in adult rat myocytes that the protection induced by diazoxide against hypoxia-reoxygenation injury, was dependent on mitochondrial ROS production, assessed using dichlorofluorescin fluorescence.([116]) This effect of diazoxide was abolished in the presence of the mitochondrial K_{ATP} channel blocker, 5-HD, and the free radical scavengers, N-acetylcysteine or N-mercaptopropionylglycine.([116]) In this paradigm, the opening of the mitochondrial K_{ATP} channel results in the production of ROS from the electron transport chain, which are released into the cytosol, where they activate downstream mediators of the preconditioning signal such as PKC.([49;117-119])

However, a recent study has demonstrated that the opening of the mitochondrial K_{ATP} channel using diazoxide may actually reduce ROS production prior to the index ischaemic period, which is in direct contrast to the findings of studies implicating a trigger role for ROS in preconditioning.([120]) However, this study was conducted on isolated mitochondria, whereas the studies demonstrating the production of ROS with diazoxide were undertaken in myocytes, which may explain the disparity in findings. In another study, again using isolated mitochondria,

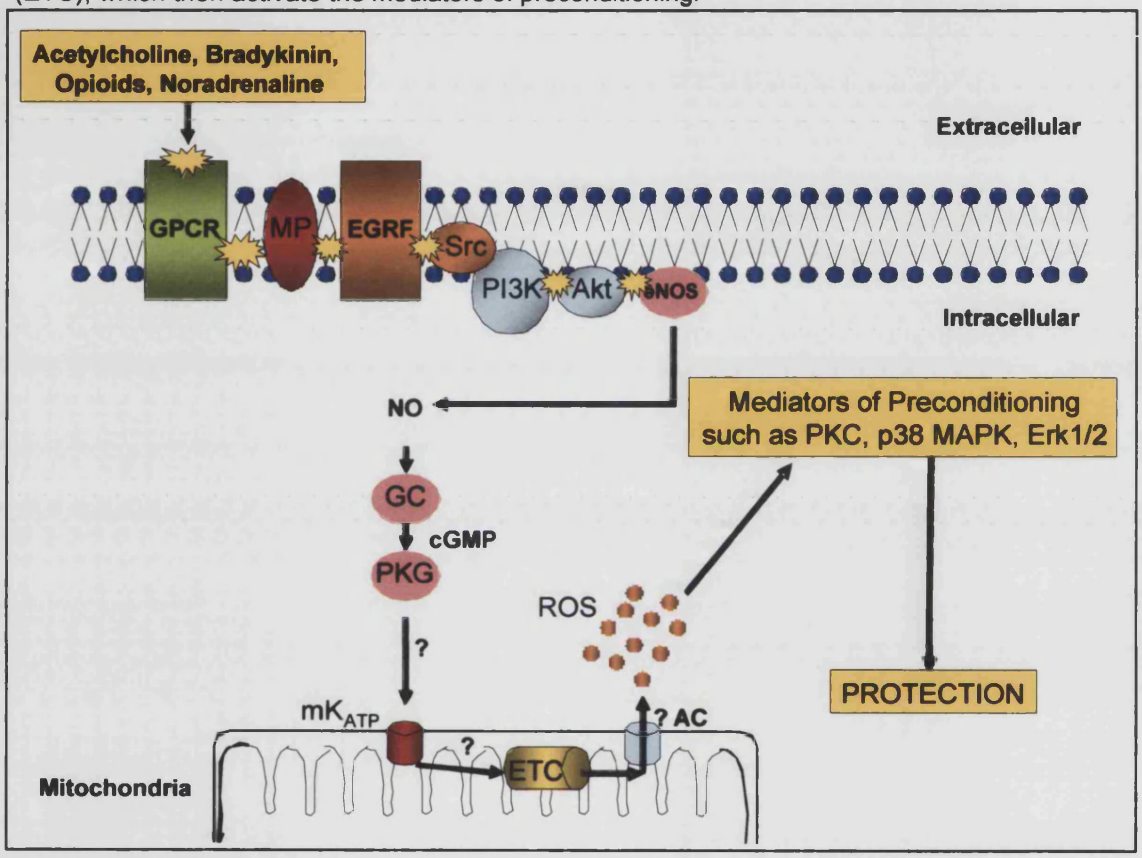
Terzic's group demonstrated that diazoxide reduced mitochondrial ROS release from complex II of the electron transport chain.([121])

Downey's group,([73;74;84;85;90]) have extensively studied the signal transduction pathways that mediate the preconditioning stimulus applied at the GPCR to the mitochondrial K_{ATP} channel (figure 1.2 is a cartoon representation of their current working model). They and others have demonstrated that the activation of the GPCR, using either acetylcholine, opioids, bradykinin or noradrenaline opens the mitochondrial K_{ATP} channel and generates mitochondrial ROS.([73;90]) Activation of the GPCR has been demonstrated to activate the epidermal growth factor receptor (EGFR),([85]) via a matrix metalloproteinase. The EGFR in conjunction with Src tyrosine kinase,([74;84;85]) then activates the PI3K-Akt pathway.([74;80;85]) Activation of Akt then phosphorylates and activates endothelial nitric oxide synthase (eNOS),([90]) which activates guanylate cyclase,([90]) via nitric oxide. Guanylate cyclase then activates protein kinase G (PKG),([90]) via (cyclic guanine-5-monophosphate) cGMP. Finally, PKG via some unknown mechanism opens the mitochondrial K_{ATP} channel,([90]) which results in the mitochondrial release of ROS that then activate the mediators of preconditioning such as PKC (see figure 1.2).

The requirement for nitric oxide (NO) as a trigger in classical preconditioning suggested by the scheme in figure 1.2 is controversial with some studies demonstrating a role for NO([122-124]) but others do not, including a study by Downey's own group.([125]) The discrepancy has been attributed to the concentration of N ω-nitro-L-arginine methyl ester (L-NAME, the nitric oxide synthase inhibitor) used in the different models.([90])

Interestingly, the activation of the GPCR by adenosine has been demonstrated by Downey's group, to not signal through this pathway but appears to activate PKC directly without the requirement for either PI3K-Akt pathway, the mitochondrial K_{ATP} channel or ROS,([73;126]) although this is in contradiction to a study by the same group which did implicate the PI3K-Akt pathway in adenosine-induced protection.([85])

Figure 1.2: The Signal Transduction Pathway Recruited by Preconditioning Triggers Which Act Via the GPCR: Activation of the G-protein coupled receptor (GPCR) leads to the trans-activation of the epidermal growth factor receptor (EGRF), which then activates the PI3K-Akt pathway in an Src kinase dependent manner. Signalling through the PI3K-Akt pathway results in the phosphorylation and activation of endothelial nitric oxide synthase (eNOS) which then activates guanylate cyclase (GC) via nitric oxide (NO). Guanylate cyclase then activates protein kinase G (PKG) via cyclic guanine-5-monophosphate (cGMP). PKG is then postulated to open the mitochondrial K_{ATP} channel (mK_{ATP}) via an unknown mechanism. Opening of the mK_{ATP} channel then results in the mitochondrial release, possibly via the inner membrane anion channel (IMAC), of reactive oxygen species (ROS), generated from the electron transport chain (ETC), which then activate the mediators of preconditioning.



Upstream Activators of the Mitochondrial K_{ATP} Channel

The hypothetical scheme, recently proposed by Downey's group, which is depicted in figure 1.2, postulates that PKG may act as the upstream activator of the mitochondrial K_{ATP} channel.([90]) In support of this proposition, Han and colleagues([127]) demonstrated that the guanylyl cyclase-cGMP-PKG pathway could phosphorylate the sarcolemmal K_{ATP} channel, and Marban's group([128]) have reported that nitric oxide can open the mitochondrial K_{ATP} channel, using flavoprotein oxidation in rabbit myocytes, to indicate channel opening.

Evidence from previous studies have postulated that PKC may be responsible for regulating the mitochondrial K_{ATP} channel in IPC.([129;130]) The role of PKC in the setting of IPC is reviewed in section 1.3.3.1.

Interestingly, Lebuffe and colleagues([124]) have recently demonstrated that ROS may contribute at multiple steps during the preconditioning phase, by activating the mitochondrial K_{ATP} channel, as well as being generated as a consequence of mitochondrial K_{ATP} channel opening. In support of this finding, an earlier study by Zhang and colleagues([131]) had demonstrated superoxide activation of bovine heart mitochondrial K_{ATP} channels reconstituted into planar lipid bilayers.

How Does Mitochondrial K_{ATP} Channel Opening Generate Mitochondrial ROS?

The mechanism through which the opening of the mitochondrial K_{ATP} channel using diazoxide or pinacidil generates mitochondrial ROS in unclear but several mechanisms have been proposed: (1) Garlid's laboratory([110;132]) have proposed that the opening of the mitochondrial K_{ATP} channel causes K^+ influx into the mitochondrial matrix coupled with H^+ efflux out of the mitochondria. The increase in matrix pH is accompanied by the influx of anions such as P_i, but because of the relatively low cytosolic concentration of P_i, the net effect of K^+ influx into the mitochondrial matrix on opening of the mitochondrial K_{ATP} channel is matrix alkanisation.([132]) In separate experiments, Garlid's group have demonstrated in isolated mitochondria that matrix alkanisation can increase ROS production.([133]) However, it has not been demonstrated directly that opening of the mitochondrial K_{ATP} channel generates mitochondrial ROS via causing matrix alkanisation. (2) Inhibition of mitochondrial respiration generates ROS from the electron transport chain (ETC).([134]) During the brief ischaemic episode of an IPC stimulus one may expect that the transient inhibition of the ETC that occurs may be sufficient to generate ROS. Furthermore, the short reperfusion period that is required as part of the IPC-stimulus, may be expected to further produce ROS, by re-oxygenating a mildly inhibited ETC. In this regard, it is interesting to note that some of the pharmacological preconditioning agents such as diazoxide and pinacidil that are believed to generate ROS via the opening of the mitochondrial K_{ATP} channel, can inhibit the ETC at complex II.([78;135;136]) Therefore, the wash-out of the preconditioning agent (which forms part of the experimental protocol when using these drugs) could potentially release the inhibitory effect on the ETC, and generate ROS, without invoking the opening of the mitochondrial K_{ATP} channel. This issue is discussed in section 1.3.2.4.d, which addresses some of the controversies surrounding the mitochondrial K_{ATP} channel, which have arisen from not knowing the molecular identity of the channel.

1.3.2.4.c The Identity of the Mitochondrial K_{ATP} Channel

Despite being described nearly 14 years ago,([102]) and despite being thoroughly characterised by Garlid's group,([103;132;137-139]) the molecular composition of the mitochondrial K_{ATP} channel remains unknown. Because the cardiac sarcolemmal K_{ATP} channel was demonstrated to comprise the Kir6.2 (about 40 kDa) and SUR2A (about 140 kDa) subunits,([93;140]) the initial attempts at identifying the mitochondrial K_{ATP} channel were aimed at detecting Kir or SUR subunits within mitochondrial membranes. Suzuki and colleagues([141]) demonstrated that mitochondria immuno-stained with antibodies, that had been generated to bind to part of the Kir6.1 subunit. However, evidence from Garlid's group([142]) found that this antibody did not react with any protein in the reconstitutively active purified fraction of the mitochondrial K_{ATP} channel. Furthermore, Marban's group([143]) demonstrated that rabbit myocytes with dominant negative constructs of Kir6.1 and Kir6.2, still possessed mitochondrial K_{ATP} channel activity as determined by changes in flavoprotein oxidation. Binding studies have failed to identify a conventional SUR within mitochondrial membranes, suggesting instead that mitochondrial SUR may be smaller proteins ranging from 28 to 63 kDa in size.([142;144]) Garlid's group([142]) have identified a 55 kD channel protein (which they have termed mitoKir) and a 63 kDa sulphonylurea receptor (which they have termed mitoSUR) as potential components of the mitochondrial K_{ATP} channel. They([142]) have postulated that the 63 kDa protein, may turn out to be an half ABC protein, called mitochondrial ATP binding cassette protein-2 (M-ABC2), which has been identified in mitochondrial membranes and proposed to be part of the mitochondrial K_{ATP} channel.([145])

Recently, Marban's group([146]) have proposed a new model for the mitochondrial K_{ATP} channel, using a novel approach to identifying its components. Based on the premise that diazoxide, an opener of the mitochondrial K_{ATP} channel, also inhibits succinate dehydrogenase (SDH), using pull-down columns they determined which mitochondrial membrane proteins were complexed to SDH.([146]) Interestingly, they identified adenine nucleotide translocase (ANT), an ATPase, M-ABC1 and a phosphate carrier. They demonstrated that K^+ fluxes through this complex were regulated by diazoxide and 5-HD. However, they could not identify which of these components formed the K^+ conductance pore. Interestingly, the ANT has also been proposed to form part of the mitochondrial permeability transition pore (mPTP), discussed in section 1.5. It is intriguing to speculate on the possibility that the mPTP may be masquerading as the mitochondrial K_{ATP} channel. Against the ANT forming the K^+ conductance pore of this new

proposed model of the mitochondrial K_{ATP} channel, the complex was demonstrated to be insensitive to atractyloside, which would have been expected to change the ANT into a pore-forming configuration, if the ANT had been responsible for the K^+ conductance property.([146])

To add to the confusion over the identity of the mitochondrial K_{ATP} channel, Brian O'Rourke's group([147]) have recently identified a Ca^{2+}-activated K^+ channel in cardiac inner mitochondrial membrane that can mediate protection against ischaemia-reperfusion injury.

Therefore, because the molecular composition of the mitochondrial K_{ATP} channel still remains unknown, the evidence concerning its existence and actions have been inferred by the pharmacological manipulation of the channel using agents such as diazoxide and 5-HD, which not surprisingly has generated the controversy surrounding the channel's actual existence, a subject which is reviewed in the next section.

1.3.2.4.d The Controversies Surrounding the Mitochondrial K_{ATP} Channel

The Non-Specific Effects of Drugs Used to Investigate the Mitochondrial K_{ATP} Channel

The controversies that surround the mitochondrial K_{ATP} channel relate to the fact that the molecular identity of the mitochondrial K_{ATP} channel is unknown. Therefore, the existence of the channel can only in the most part be inferred by the presumed actions of pharmacological agents on the channel. However, these drugs, which include diazoxide, pinacidil and 5-hydroxydecanoic acid have been demonstrated to exert effects on mitochondria, independent of their effect on the mitochondrial K_{ATP} channel, which may in part explain their capacity to influence IPC-induced protection.

For example, diazoxide and 5-hydroxydecanoic acid (5-HD) have been demonstrated to be a specific opener and blocker of the mitochondrial K_{ATP} channel, respectively.([104;106;108;138]) However, diazoxide has been demonstrated to inhibit succinate dehydrogenase, which forms part of the FADH-linked electron transport carrier II,([107;121;135;136;148;149]) and may induce cardio-protection independent of the mitochondrial K_{ATP} channel.([78;150]) Terzic's group demonstrated that diazoxide inhibits SDH with an IC_{50} of 32 μmol/l.([121]) Inhibition of SDH could also account for the observed flavoprotein oxidation observed with diazoxide by Marban's group.([108])

These studies would suggest that the inhibition of the ETC may induce preconditioning-like protection. Interestingly, Kukreja's group([151]) showed that inhibiting SDH, using low dose 3-nitropropionic acid (3-NPA, an irreversible inhibitor of SDH), protected in vivo rabbit hearts from infarction, an effect abolished by 5-HD. This study demonstrated that inhibiting SDH directly

could mediate cardio-protection, a finding supported by Terzic's group,([78]) who demonstrated that the SDH inhibitor, malonate, could mimic the protective effect induced by either diazoxide or nicorandil against ischaemia-reperfusion injury. Furthermore, they demonstrated that the K^+ ionophore, valinomycin could also mimic protection, suggesting that a non-specific K^+ conductance was sufficient to induce preconditioning-like protection.([78]) In neuronal tissue, chemical preconditioning using 3-NPA has been demonstrated in several studies to be neuro-protective.([152-156])

The mechanism by which the inhibition of SDH induces cardio-protection may be via mitochondrial ROS release, which may then mediate the protection observed with these preconditioning mimetics (see section 1.3.2.2). In this regard, the SDH inhibitor, 3-NPA, has been shown to induce a burst of free radicals, albeit in cerebral tissue.([157])

In addition, pinacidil, another cardio-protective agent was demonstrated to inhibit NADH oxidation.([107]) Hanley and colleagues also demonstrated that 5-HD, the presumed archetypal mitochondrial K_{ATP} channel blocker, is converted to 5-HD-CoA, the β-metabolites of which can cause metabolic effects which can antagonize the beneficial effects of preconditioning.([107;149;158]) This may provide a mitochondrial K_{ATP} channel-independent mechanism by which 5-HD antagonises the effects of diazoxide and pinacidil, suggesting that these drugs can modify IPC-induced protection independent of the mitochondrial K_{ATP} channel.

The Method for Detecting Mitochondrial Swelling

Another controversial issue is the effect of diazoxide and 5-HD on mitochondrial matrix volume as assessed by light scattering. Garlid's group([159;160]) have demonstrated that diazoxide-induces an ATP-sensitive decrease in light scattering, indicating mitochondrial matrix swelling secondary to a K^+ influx. However, Halestrap's group([161]) were unable to demonstrate a diazoxide or 5-HD induced change in mitochondrial matrix volume, by measuring light scattering. Instead they attributed the changes in light scattering observed by Garlid's group on ATP/ADP-induced conformational changes in mitochondria rather than changes in volume. Therefore, both groups agree that diazoxide induces a K^+-dependent increase in mitochondrial matrix volume, but the dispute revolves around the technique used for demonstrating this increase, and whether the mitochondrial matrix expansion is mediated by an ATP-sensitive K^+ channel or not.([149;159-161])

1.3.2.4.d The Renaissance of the Sarcolemmal K_{ATP} Channel

While various laboratories continue to debate the role of the mitochondrial K_{ATP} channel in ischaemic preconditioning, several other groups have been re-investigating the role of the sarcolemmal K_{ATP} channel as a component of preconditioning-induced protection. The contribution of this channel to ischaemic preconditioning had been more or less overlooked since its mitochondrial counterpart entered the scene in 1997,([104;108]) and it was demonstrated that agents which opened cardiac K_{ATP} channels were able to protect the heart without affecting the APD.([50;100;101])

However, several studies have been published demonstrating that the protection associated with opening of the sarcolemmal K_{ATP} channel was dependent on APD shortening.([97-99]) Terzic's group([162;163]) have demonstrated that transfection of K_{ATP}-deficient cells with Kir6.2/SUR2A (components of the sarcolemmal K_{ATP} channel) genes resulted in a pinacidil sensitive K^+ current and mediated protection against hypoxia-reoxygenation injury, implicating a role for the sarcolemmal K_{ATP} channel in cardio-protection.

A recent study published in 2002 by Suzuki and colleagues([164]) provides strong evidence that the sarcolemmal K_{ATP} channel is important for IPC-induced protection. Using transgenic mice lacking Kir6.2 (a component of the sarcolemmal K_{ATP} channel), these investigators failed to demonstrate a reduction in infarct size with IPC.([164]) However, the fast-beating murine heart is likely to be more sensitive to changes in APD when compared to other animal models. Rajasharee and colleagues([165]) demonstrated that isolated perfused murine hearts, taken from mice expressing a mutant Kir6.2 subunit, were insensitive to IPC, using protection against myocardial stunning as the end-point. However, in the isolated perfused heart, IPC may reduce infarct size without improving the recovery of contractile function.([166])

A further study by the same group demonstrated that diazoxide had no protective effect against myocardial stunning in transgenic mice lacking Kir6.2, and that the diazoxide-induced protection provided in wild-type mice was associated with APD shortening and was blocked by HMR1098 (the specific sarcolemmal K_{ATP} channel blocker), but not 5-HD (a presumed mitochondrial K_{ATP} channel blocker).([167]) A recent study by Terzic's group([168]) demonstrated that the inability to ischaemically precondition the hearts from Kir6.2-knockout mice was related to the critical role the sarcolemmal K_{ATP} channel plays in mediating preservation of myocardial energetics during ischaemia-reperfusion.

Therefore, these recent studies add further confusion to the role of K_{ATP} channels in IPC, and suggest a role for the sarcolemmal K_{ATP} channel in IPC-induced protection. This confusion is compounded by studies suggesting that both channel types may be involved in IPC.([169-171])

1.3.3 Mediators (Signal Transduction) of Classical Preconditioning

1.3.3.1 Protein Kinase C

Studies by Ytrehus and colleagues in the rabbit,([172]) and Yellon's group in the rat,([173]) were the first to demonstrate that the activation of protein kinase C (PKC) was required for IPC-induced protection, using PKC analogues such as phorbol 12-myristate 13-acetate, and diacylglycerol to induce protection. PKC is a serine/threonine kinases of which there are 12 isoforms divided into 3 groups: conventional (α, β_I, β_{II} and γ), novel (δ, ε, η, θ) and atypical (ξ, ι, λ, μ).([174])

Later studies demonstrated that PKC was a downstream element of the signal transduction pathway of preconditioning, since the protection associated with preconditioning triggers such as bradykinin,([55]) adenosine,([61]) opioids,([62]) and ROS,([63]) could all be blocked by PKC inhibitors. Furthermore, the redundancy effect observed with the GPCR ligands in mediating IPC-induced protection could be correlated to the extent of kinase activity of PKC.([55])

Activation of PKC requires the translocation from the cytosol to the particulate fraction which includes the membranes and cytoskeleton. Translocation is dependent on isoform-specific binding of PKC to receptors of activated C kinase (RACKs).([175;176]) Localisation of the isozyme specific PKC-RACK complex can determine different cellular functions. It has been demonstrated that IPC induces the translocation of PKC-δ and PKC-ε in rats hearts.([177-179]) Ping and colleagues demonstrated translocation of PKC-ε and PKC-η in rabbit hearts.([180])

The role of PKC as a mediator of IPC-induced protection was suggested by Downey's group([181]) when they demonstrated that IPC-induced protection was only abolished by the PKC inhibitor, staurospaurine, if the latter was given during the index ischaemic period, and not if it was given during the preconditioning phase. The same group postulated that it was the relatively slow translocation of PKC induced by the preconditioning triggers, which provided the memory of IPC.([182]) Further studies using isozyme-specific inhibitors for PKC-ε, have implicated this particular isozyme in IPC-induced protection at least in rabbit hearts.([183])

Interestingly, Mochly-Rosen's group([184;185]) have recently demonstrated that the PKC-δ isoform may contribute to ischaemia-reperfusion injury. They demonstrated that inhibiting the

PKC-δ isoform, protected the isolated perfused rat heart against ischaemia-reperfusion injury.([184]) Furthermore, they demonstrated that the protection associated with the inhibition of PKC-δ and the activation of PKC-ε was additive.([185]) Further studies by the same group in the open chest pig demonstrated that PKC-δ may act as a mediator of lethal reperfusion injury.([184;185])

The mechanism by which PKC induces cardio-protection is unclear but it may relate to either their activation of the mitogen-activated protein kinases (see section 1.3.3.3), or the mitochondrial K_{ATP} channel.([129;130]) Another mediator of IPC-induced protection is tyrosine kinase which is reviewed next.

1.3.3.2 Tyrosine Kinase

Tyrosine kinases can be divided into: (1) receptor tyrosine kinases which may act as preconditioning triggers by activating PKC and (2) cytosolic receptor tyrosine kinases which may act as preconditioning mediators by acting downstream or in parallel with PKC. Activation of receptor tyrosine kinases can take place in response to GPCR-ligand binding.([186]) Recent studies by Downey's group suggest that Src tyrosine kinase in conjunction with the epidermal growth factor receptor may be required to activate the PI3K-Akt pathway, a kinase cascade recently implicated in IPC-induced protection.([74;84;85])

Maulik and colleagues([187]) were the first to demonstrate that genistein, the tyrosine kinase inhibitor could block IPC-induced protection in rat hearts, and Downey's group([188]) determined that cytosolic tyrosine kinase activation was required during the index ischaemic period, and was likely to be downstream of PKC, at least in rabbit myocardium. Studies in swine hearts have suggested that tyrosine kinase may act in parallel to PKC,([189]) suggesting redundant parallel pathways in the porcine myocardium.

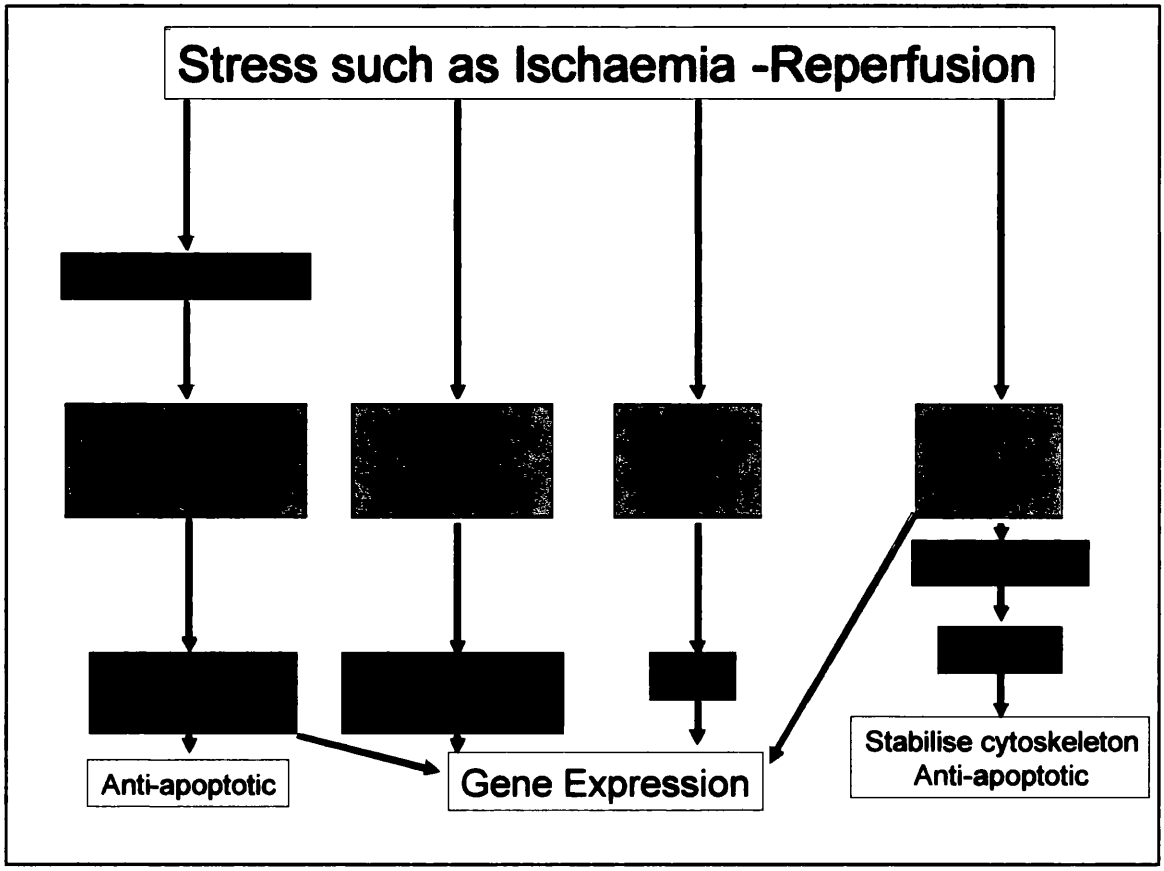
The mechanism by which the receptor tyrosine kinases induce cardio-protection is unclear but it may relate to their activation of the mitogen-activated protein kinases.

1.3.3.3 Mitogen-Activated Protein Kinases (MAPK's)

The MAPK's encompass 4 major kinase cascades in the heart, the p38 MAPK, the c-Jun NHP_2 terminal kinase (JNK), the 42 and 44-kDa extracellular signal-regulated (Erk1/2) MAPK, and the big MAP kinase 1 (BMK1 or Erk5).([190-192]) These 4 major kinase pathways exhibit the same conserved three-tier module of a MAPKKK/MKKK (MEKK) phosphorylating a MAPK kinase

big MAP kinase 1 (BMK1 or Erk5).([190-192]) These 4 major kinase pathways exhibit the same conserved three-tier module of a MAPKKK/MKKK (MEKK) phosphorylating a MAPK kinase (MKK or MEK) which then activates, the MAPK which comprises the JNK, p38, BMK1 or Erk1/2 MAP kinases. These kinase cascades are activated in response to stress such as ischaemia-reperfusion and receptor protein tyrosine kinases, GPCR's and PKC (see figure 1.3 for scheme).([192])

Figure 1.3: Schematic Representation of the four Major Classes of MAPK Family. Each major class of MAPK, Erk1/2, BMK1, JNK1/2 and p38 comprises a well-conserved three tier module of a MKKK-MKK-MAPK. These MAPK's are activated in response to ischaemia-reperfusion and can be activated in response to an IPC stimulus (with G-protein coupled receptors, GPCR's or receptor tyrosine kinases, RTK's). These MAPK's phosphorylate their substrates (in green-see text for details) which mediate protection recruiting anti-apoptotic protective mechanisms or regulating gene expression.



Erk1/2 MAP Kinase

Whether Erk1/2 MAPK contributes to cardio-protection associated with classical preconditioning is unclear, with studies demonstrating that it is activated prior to the index ischaemic period,([82;193-195]) but not all of them show these MAPK's contributing to IPC-induced protection.([193;194]) In contrast there are studies reporting no change in Erk1/2 activity in the setting of IPC.([196;197]) A recent study suggests that diazoxide-induced mitochondrial ROS release may activate Erk1/2.([75]) This is interesting given the findings of a study by Baines and colleagues,([198]) which demonstrated that PKC-ε modules can form complexes with Erk1/2-BAD, p38 and JNK at the mitochondria. In this scenario, PKC-ε phosphorylated both Erk1/2 and p38 MAPK but down-regulated JNK.([198]) Erk1/2 can mediate cellular protection by phosphorylating recruiting several anti-apoptotic mechanisms (see section 1.4.3).

p38 MAP Kinase

The role of p38-MAPK in IPC-induced protection is also controversial with studies demonstrating increased activation of p38-MAPK during the index ischaemia in preconditioned isolated rabbit and rat hearts,([174;199-201]) and rabbit cardiomyocytes,([202]) as well as activation of MAPKAPK2, a downstream substrate of p38-MAPK.([203]) In contrast, several studies have reported that the p38-MAPK activation that occurs during the index ischaemia is transient and does not correlate with the preconditioning effect.([196;204]) To complicate matters further, studies have also shown that p38-MAPK activity is attenuated in preconditioned rabbit([205]) and rat hearts,([206;207]) suggesting that p38-MAPK activation is pro-injurious, a finding which is supported by studies demonstrating that inhibiting p38-MAPK, using SB203580, protects the heart against ischaemia-reperfusion injury.([208-210]) In contrast other studies have demonstrated that inhibiting p38-MAPK abrogated preconditioning-induced protection,([200;211]) but only if the p38-MAPK inhibitor was present during the index ischaemic episode.([212])

The discrepancy in results may be attributed in part to the different p38-MAPK isoforms that exist, with p38α contributing to myocyte death, and p38β responsible for cell survival.([195;213]) Marber's group([195]) have demonstrated that preconditioning decreased p38α MAPK activity during the index ischaemia, suggesting that this isoform was responsible for mediating ischaemia-reperfusion injury. In further studies Marber's group([214]) demonstrated that the nitric oxide donor, S-nitro N-acetyl penicillamine (SNAP) could delay the ischaemia-induced activation of p38α in myocytes subjected to hypoxia-reoxygenation.

JNK MAP Kinase

The JNK MAPK family comprise two isoforms, the 46 kDa JNK1 and the 54 kDa JNK2, both of which are present in the heart.([215]) Clerk and colleagues([215;216]) have demonstrated that both JNK1 and JNK2 are activated upon reperfusion but are not affected by ischaemia, whereas other reports suggested that ischaemia-reperfusion may also activate JNK.([217;218]) In the context of IPC, Takeishi and colleagues([197]) found that IPC resulted in a more rapid activation of JNK during the index ischaemic period, although the significance of this to IPC-induced protection is unclear. Iliodromitis and colleagues([201]) found that IPC induced an increase in both JNK1 and JNK2, confirming the findings of Ping and colleagues([219]) who also demonstrated a PKC-dependent activation of both JNK's during IPC in conscious rabbits. In contrast, Downey's group reported no change in JNK activation when isolated rabbit hearts were subjected to IPC.([203])

Big MAP Kinase

Recently a new member of the MAPK family has been described, called the big MAP kinase (BMK1 or Erk5).([190;191]) This MAPK is anti-apoptotic and it may mediate cellular responses by regulating expression of the gene c-jun,([220;221]) the expression of which has been shown to occur during ischaemia or hypoxia.([218;222]) BMK1 is activated by MEK5 and also results in the phosphorylation of MEF2A and MEF2C, transcription factors that belong to the myocyte enhancing factor-2 (MEF2) family, which are involved in cardiac gene expression.([221])

Takeishi and colleagues([223]) have demonstrated activation of this MAPK during ischaemia reperfusion, and have reported that preconditioning in rabbit hearts increased the ischaemic-induced activation of BMK1,([197]) though the importance of this MAPK to protection is unclear at present.

In summary, the contribution of the MAPK's to IPC-induced protection is shrouded with confusion. Disparity between the different studies may in part be due to presence of different isoforms with contrasting actions or the use of different experimental model and species.

1.3.3.4 The JAK-STAT Pathway

The Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway is a stress-responsive mechanism that transduces signals for the cell membrane to the nucleus, where gene expression is modulated (reviewed in reference ([224])). The JAK-STAT pathway is

activated in response to ischaemia-reperfusion and has been demonstrated to mediate apoptosis (STAT1)([225]) and myocardial dysfunction (STAT5A, STAT6).([226]) In contrast STAT3 was demonstrated to contribute to IPC-induced protection in a study by Hattori and colleagues.([227]) They found that IPC produced an early activation of JAK2 and STAT3 in isolated rat hearts.([227]) IPC reduced infarct size and attenuated apoptotic cell death concurrent with up-regulation of the anti-apoptotic protein BCl_2 and down-regulation of the pro-apoptotic protein BAX. The presence of the JAK inhibitor, AG-490 abolished JAK2 and STAT3 phosphorylation and abrogated the IPC-mediated reduction in infarct size and apopotosis, suggesting an important role for STAT 3 in IPC-induced protection.([227])

1.3.4 End-Effectors of Classical Preconditioning: Mechanisms of Protection

1.3.4.1 Mitochondrial Function

The survival and recovery of myocytes following an episode of ischaemia-reperfusion injury depends critically on maintaining mitochondrial function during this insult. Therefore, optimizing mitochondrial function with respect to energy production and ionic homeostasis is crucial if myocytes are to survive ischaemia-reperfusion. Murry and colleagues([5]) in the first study describing IPC, postulated that the protection-induced by IPC may be the result of reduced ATP depletion and/or reduced catabolite accumulation.

Later studies have demonstrated that IPC protects by: (1) Attenuating ATP hydrolysis during ischaemia;([228]) (2) Increasing creatine phosphate and reducing ATP consumption;([229-231]) (3) Reducing demand for high energy phosphates because of lower mitochondrial ATPase activity;([232]) (4) Preserving mitochondrial energy production at reperfusion;([231;233]) and (5) Increasing ATP synthesis.([234]) In contrast, Kolocassides and colleagues([235]) demonstrated that IPC-treated rat hearts went into contracture earlier and had lower ATP levels during the index ischaemic period.

Recent studies have demonstrated that modest uncoupling of mitochondrial oxidative phosphorylation can mimic preconditioning-induced protection.([236;237]) Furthermore, mitochondrial uncoupling has been demonstrated to be a critical event in IPC-induced protection.([150]) The mechanism by which mitochondrial uncoupling protects the heart from ischaemia-reperfusion injury is unknown but it may relate to a reduction in intracellular([238]) and

mitochondrial Ca^{2+} loading,[239] and this effect has been linked to opening of the mitochondrial K_{ATP} channel (see section 1.3.4.3).[239]

1.3.4.2 Protection Against the Apoptotic Component of Cell death

Recent advances in our understanding of cell death during ischaemia-reperfusion have implicated two forms of cell death in the pathology of a myocardial infarction, namely necrosis and apoptosis.[240] Apoptosis is a regulated, energy-dependent process which results in chromatin condensation, DNA fragmentation and apoptotic body formation, preserved cell membrane integrity, without an associated inflammatory response.[241] In contrast, necrosis is characterised by membrane disruption, massive cell swelling, cell lysis and fragmentation, with an associated acute inflammatory response. The exact *contribution* of these two forms of cell death in the setting of ischaemia-reperfusion injury is unclear, as are the factors which determine whether the apoptotic or necrotic death pathway is recruited (this subject is discussed in section 1.4.2).

Piot and colleagues,[242] using the open chest rat model, were the first to report a reduction in the apoptotic component of myocyte death, as measured by a reduction in DNA fragmentation. Later studies have confirmed this finding, using the same model, concurrent with a reduction in the pro-apoptotic protein, BAX expression.[243] A reduction in apoptosis has also been reported in isolated perfused rat hearts,[244;245] with an associated increase in the anti-apoptotic protein, B-cell lymphoma 2 gene (BCl_2).[244]

IPC may reduce the apoptotic component of cell death by: (1) inhibiting the generation of ROS;[246] (2) preserving vascular endothelium;[247;248] (3) up-regulating the anti-apopototic protein BCl_2,[244] or down-regulating the pro-apoptotic proteins BAX,[243] and p53;[249;250] (4) reducing mitochondrial cytochrome C release;[239;251;252] (5) reducing caspase activity;[253;254] or by (6) opening of the mitochondrial K_{ATP} channel.

1.3.4.3 The Mitochondrial K_{ATP} Channel

The finding that mitochondrial K_{ATP} channel opening could protect the heart against ischaemia-reperfusion injury[104;108] and that 5-hydroxydecanoic acid (the presumed mitochondrial K_{ATP} channel blocker) could abolish IPC-induced protection,[95;96] led investigators to propose the mitochondrial K_{ATP} channel as the mediator/end-effector of IPC-induced protection,[50;104;108] with several studies demonstrating that the mitochondrial K_{ATP} channel has to be open during

the index ischaemic period.([169;255;256]) Several hypotheses have been proposed to account for the protection induced by mitochondrial K_{ATP} channel opening, and include the following:

1.3.4.3.a Reduction in Mitochondrial Ca^{2+} Load due to Mitochondrial Membrane Depolarisation

Accumulation of mitochondrial Ca^{2+} during ischaemia-reperfusion is detrimental to mitochondrial function.([21;22]) Studies have suggested that mitochondrial K_{ATP} channel opening can reduce mitochondrial Ca^{2+} loading, by inducing mitochondrial membrane depolarisation, which would in turn reduce the electrochemical gradient for Ca^{2+} entry.([257;258]) However, these studies have attracted controversy because of the high concentrations of diazoxide and pinacidil used, and over whether mitochondrial K_{ATP} channel opening actually induces a significant mitochondrial membrane depolarisation.([109;159;160;259]) At high concentrations, diazoxide and pinacidil can uncouple mitochondria, independent of mitochondrial K_{ATP} channel opening.([160;260]) In support of an uncoupling action being critical for preconditioning-induced protection, Sack's group([150]) have demonstrated that diazoxide protects by inducing mitochondrial uncoupling. Furthermore, pharmacological-induced mitochondrial uncoupling can mimic preconditioning-induced protection.([236;237])

Studies in other laboratories have noted that IPC and diazoxide protect against ischaemia-reperfusion injury by causing reduction in mitochondrial Ca^{2+} load.([52;239;261;262]) Crestanello and colleagues([263-265]) found that IPC and mitochondrial K_{ATP} channel openers preserved mitochondrial function and rendered mitochondria more tolerant to mitochondrial Ca^{2+} loading, compared to control mitochondria.

However, one problem with this suggested role for mitochondrial K_{ATP} channel opening is that during myocardial ischaemia, the mitochondrial membrane potential is reduced, and so the role of mitochondrial K_{ATP} channel openers in reducing mitochondrial calcium loading by depolarising the mitochondrial membrane potential may not be of great significance. This would suggest an alternative mechanism for reducing calcium load during ischaemia. The mitochondrial permeability transition pore (mPTP) has been demonstrated to act as a mitochondrial Ca^{2+} release channel, and some studies have demonstrated that diazoxide may induce mitochondrial Ca^{2+} efflux via this route (see section 1.5.6.3.a).([257;258;266])

1.3.4.3.b Improved Energy Production During Ischaemia-Reperfusion

Preserving mitochondrial energy production is vital to myocyte survival during ischaemic-reperfusion injury. Opening of the mitochondrial K_{ATP} channel has been demonstrated to increase ATP synthesis,([234]), preserve mitochondrial energy production,([267]) decrease ATP hydrolysis,([159]) and improve energy transfer at reperfusion.([159])

Garlid's laboratory([159]) have proposed that during myocardial ischaemia, the opening of the mitochondrial K_{ATP} channel increases mitochondrial matrix volume thereby preserving the integrity of the intermembranous space which facilitates energy transfer and reduces ATP hydrolysis.

At the time of reperfusion, opening of the mitochondrial K_{ATP} channel preserves the impermeability of the mitochondrial outer membrane to cytochrome C and adenine nucleotides.([159]) In support of a role for mitochondrial K_{ATP} channel opening at the time of reperfusion, several studies have demonstrated that activating the mitochondrial K_{ATP} channel at reperfusion can protect the myocardium from lethal reperfusion injury([79;169;268;269]) Other studies however, have failed to show cardio-protection with the pharmacological activation of the mitochondrial K_{ATP} channel using either diazoxide([256]) or nicorandil.([270;271])

1.3.4.3.c Reduced Apoptotic Cell Death

Ashraf's group([272]) have demonstrated that mitochondrial K_{ATP} channel opening can reduce the apoptotic component of myocyte death following ischaemia-reperfusion injury. The same group demonstrated that this protective effect was related to the ability of mitochondrial K_{ATP} channel opening to maintain the mitochondrial membrane potential following a simulated episode of ischaemia-reperfusion.([273]) This finding was confirmed by Marban's group using the purported mitochondrial K_{ATP} channel openers, diazoxide,([274]) pinacidil([274]) or nicorandil.([275]) Garlid's group([159]) demonstrated that mitochondrial K_{ATP} channel opening could preserve the impermeability of the outer mitochondrial membrane to cytochrome C during ischaemia-reperfusion, thereby preventing the translocation of mitochondrial cytochrome C into the cytosol and avoiding apoptosis.

1.3.4.4 Reducing the Generation of Reactive Oxygen Species

Reactive oxygen species generated on reperfusion of ischaemic myocardium, are a major cause of lethal reperfusion injury (see section 1.4.1.1).([79;276;277]) Therefore reducing oxidative

stress at the time of reperfusion offers protection against lethal reperfusion injury. In this regard, Crestanello and colleagues([278]) were the first to demonstrate that IPC reduces the production of ROS, as measured by chemiluminescence, at the time of reperfusion. Vanden Hoek's group([79]) reported in chick myocytes that preconditioning using either hypoxia or adenonsine protected the cells by reducing oxidative stress at reoxygenation, a finding that has been confirmed by another group.([77]) The K_{ATP} channel openers, diazoxide and nicorandil have also been demonstrated to protect by attenuating ROS production at the time of reoxygenation, using isolated mitochondria.([78]) Interestingly, the protective effect was also mimicked by malonate (an inhibitor of succinate dehydrogenase) and the K^+ ionophore, valinomycin, suggesting that the protective effects of these drugs were independent of their opening of a K_{ATP} channel (see section 1.3.2.4.d). The mechanism by which preconditioning results in less oxidative stress at the time of reperfusion is unknown, but Terzic and colleagues speculate that it is due to the reduction in electron flow into the coenzyme Q cycle,([78]) a critical component in ROS generation.

1.3.4.5 Gap Junctions

Gap junctions in the myocardium, which comprise connexin (Cx) 43,([279]) interconnect myocytes into a functional syncytium. During ischaemia, dephosphorylation of Cx43 occurs which allows communication between the cells via these junctions. During reperfusion, gap junction communication allows cell-to-cell spread of Na^+ which mediates hypercontracture and cell death,([280;281]) and therefore, inhibiting this communication using heptanol, a gap-junction inhibitor, can reduce cell death.([280])

A recent study has demonstrated that transgenic mice deficient in Cx43 could no longer be protected by IPC, suggesting the requirement for gap junctions to mediate IPC-induced protection.([282]) In contrast, Saltman and colleagues([283]) have demonstrated that closing gap junctions can mimic IPC-induced protection, presumably by preventing the propagation of hypercontracture and cell necrosis via the gap junctions. In support of this study, Schulz and colleagues([284]) have demonstrated that in anaesthetised pigs, IPC can phosphorylate Cx43 (keeping gap junctions closed) via p38 MAP kinase and PKC. Another study confirms that IPC maintains the phosphorylation state of Cx43 via the K_{ATP} channel.([285]) In direct contrast, another study suggests that opening gap junctions are essential for IPC-induced protection, as a possible route for some unknown 'survival' factor.([286])

1.3.4.6 The Na^+-H^+ Exchanger

The activation of the Na^+-H^+ exchanger (NHE)([287]) in response to an increase in intracellular pH during the index ischaemic period (via the activation of NHE kinases such as Erk1/2 MAP kinase)([288]), is detrimental to cellular and mitochondrial function as it results in both cytosolic and therefore mitochondrial Ca^{2+} loading. Therefore, inhibiting the Na^+-H^+ exchanger using either amiloride([289]) or a class of NHE inhibitors([290]) during ischaemia-reperfusion offers a potential target for cardio-protection and can preserve cellular and mitochondrial ionic homeostasis at this crucial time.([291;292]) In this regard, several studies have reported protection against ischaemia-reperfusion injury with this class of drugs and demonstrated that these drugs are most effective when given prior to ischaemia or after the onset of ischaemia,([291;292]) and in some cases the protection demonstrated with this class of drugs may be greater than ischaemic preconditioning.([293;294])

1.3.5 The Second Window of Protection (Delayed Preconditioning)

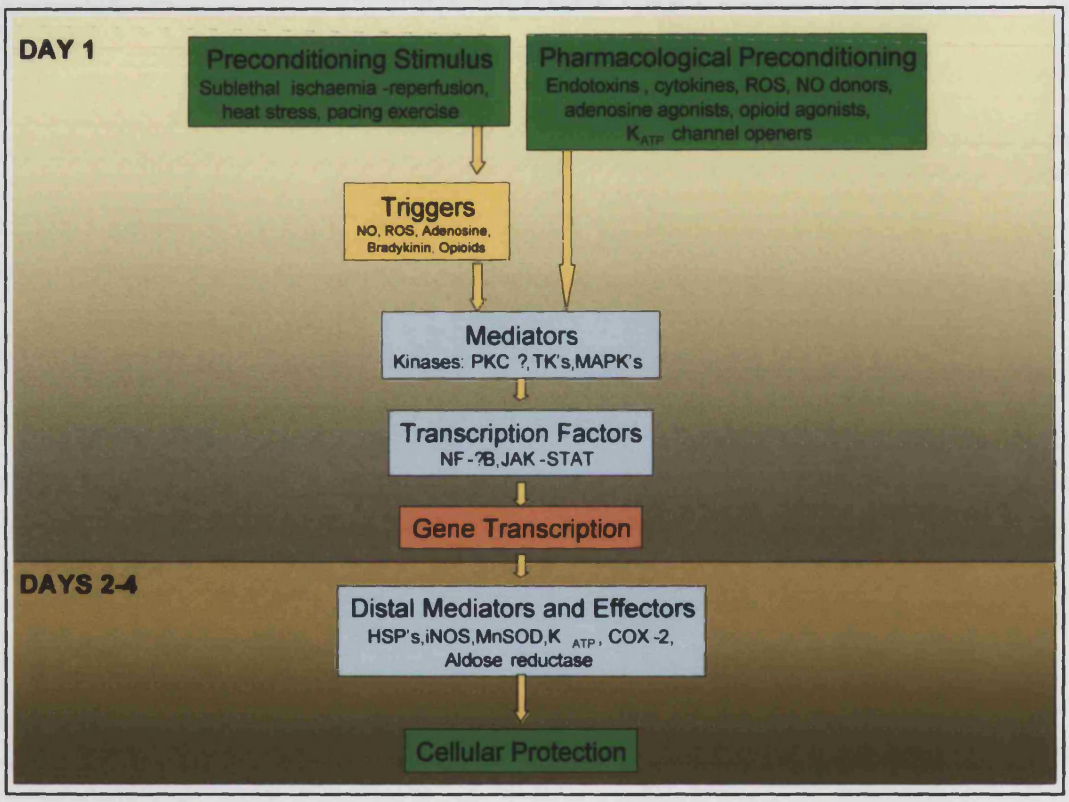
The protection induced by ischaemic preconditioning the myocardium is a biphasic phenomenon with an early phase of protection (classical preconditioning) that develops within minutes and lasts 2 to 3 hours,([5]) and a delayed phase of protection (also known as delayed preconditioning, the second window of protection or late preconditioning) manifests 12-24 hours later,([44;45]) and lasts up to 72 hours.([295]) Kuzuya and colleagues([45]) demonstrated using the open-chest dog model, that four-5 minute cycles of left anterior descending (LAD) arterial occlusion protected the heart subjected to sustained lethal ischaemia-reperfusion injury, as measured by a reduction in infarct size, 24 hours but not 3 or 12 hours later, demonstrating that classical preconditioning wanes after 3 hours and re-appears 24 hours later. Contemporaneously and independently, Marber and colleagues([44]) demonstrated using the open-chest rabbit model that a preconditioning stimulus or sub-lethal thermal stress, protected the heart 24 hours later, concurrent with up-regulation of HSP 72, and they termed this the Second Window of Protection (SWOP).

Following the initial description of delayed preconditioning in dog([45]) and rabbit,([44]) the phenomenon has been observed in pig,([296]) mouse,([297;298]) and rat,([299]) and has been reproduced in other organ tissue, such as brain,([300]) intestine,([301]) kidney,([302]) and liver.([37]) In addition to infarct size, delayed preconditioning also protects against arrhythmias,([303]) myocardial stunning,([304]) apoptosis,([305]) and endothelial dysfunction,([306]) and has been reproduced at the cellular level in response to simulated ischaemia-reperfusion,([307-309]) including human myocytes,([310;311]) and human atrial tissue.([312])

1.3.5.1 Mechanisms of Protection in Delayed Preconditioning

Since the original description of delayed preconditioning in 1993,([44;45]) the current paradigm of delayed preconditioning conceptually classifies the components of delayed preconditioning into 'triggers' (molecular species generated during the preconditioning stimulus) that activate 'mediators' such as protein kinases, which in turn mediate the transcription of distal mediators and effectors 24-72 hours later, thereby manifesting the protection (see figure 1.4).

Figure 1.4: Schematic Representation of Components of Delayed Preconditioning. Conceptually, the components of delayed preconditioning are classified into triggers, mediators and distal mediators/effectors. Triggers of preconditioning are released in response to cellular stress. Delayed preconditioning can be mimicked by pharmacological agents such as reactive oxygen species (ROS), nitric oxide (NO) donors. These triggers or agents then activate the mediators of delayed preconditioning which include the protein kinases, protein kinase C-_ (PKC_), tyrosine kinases (TK such as Src/Lck), mitogen activated protein kinases (MAPK'S). These kinases then activate transcription factors such NF-_B and the JAK-STAT pathway, which transcribe the distal effector mediators and proteins on days 2-4, which then mediate the protection against the index ischaemia-reperfusion episode. These distal mediators and effectors include heat shock proteins (HSP's), inducible nitric oxide synthase (iNOS), manganese superoxide dismutase (MnSOD), K_{ATP} channels.



1.3.5.2 Triggers of Delayed Preconditioning

1.3.5.2.a Adenosine

Adenosine, released in response to the sublethal episode of ischaemia-reperfusion that comprises a preconditioning stimulus, may act as a trigger, initiating a myocardial phenotype more resistant to a further ischaemic insult. Yellon's group([313]) were the first to demonstrate the role of adenosine as a trigger of delayed preconditioning. Using the open-chest rabbit model, they demonstrated that the presence of a non-specific adenosine antagonist, 8-p-sulphonyphenyl theophylline (SPT) during the IPC protocol abrogated the reduction in infarct size 24 hours later. Conversely, they found that administering the adenosine A1-receptor

agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA) induced a delayed preconditioning-protective effect.([313]) Subsequent studies have confirmed the involvement of A1 adenosine receptor, and have also implicated the A3 adenosine receptor as a trigger of delayed preconditioning.([314])

1.3.5.2.b Reactive Oxygen Species (ROS)

Sun and colleagues,([315]) using the open chest pig model, demonstrated that a combination of antioxidants (MPG, OSD and catalase) abolished delayed preconditioning protection against stunning, suggesting that sublethal oxidative stress acts as a trigger of delayed preconditioning. This finding has been confirmed using: (1) different end-points of protection: infarct size,([316]) protection against arrhythmias([316]) and protection against endothelial injury;([306]) (2) different inducers of protection: heat stress,([316]) and exercise;([317]) and (3) at the cellular level.([309]) The source for the ROS is currently unknown.

1.3.5.2.c Nitric Oxide

Bolli's group were the first to demonstrate that nitric oxide (NO), which is generated during ischaemia, could act as a trigger of delayed preconditioning protection against first stunning,([318]) and then infarction.([319]) The non-specific nitric oxide synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine, abolished delayed preconditioning protection,([318]) and infusion of NO donors induced delayed cardio-protection.([320]) The endothelial NOS was implicated as the source of NO in this setting.([321]) Because NO-induced delayed cardio-protection was not abolished by ODQ (the guanylate cyclase inhibitor) but by MPG,([320]) NO is now believed to induce protection by generating a peroxynitrite anion (from NO and $\cdot O_2^-$), instead of signalling through a cGMP-dependent pathway.([320])

1.3.5.2.c Bradykinin

Yellon's group([322]) were the first to demonstrate that bradykinin could act as a trigger of delayed preconditioning. They demonstrated that administering bradykinin to a rat reduced infarct size 24 hours later via a NO-dependent mechanism. The same laboratory([296]) demonstrated that a sub-threshold IPC stimulus could be augmented by the presence of an Angiotensin Converting Enzyme Inhibitor (ACE-I, which would increase bradykinin levels) to induce delayed

preconditioning in the open-chest pig model. Whether endogenous bradykinin acts as a trigger for protection is unknown at present.

1.3.5.2.d Opioids

Pharmacological activation of the δ-opioid receptor has been demonstrated to induce delayed cardio-protection in the rat.([323])

1.3.5.3 Mediators of Delayed Preconditioning

1.3.5.3.a Protein Kinase C

Yamashita and colleagues([308]) were the first to demonstrate that inhibition of PKC using staurospaurine abolished the protection associated with hypoxic delayed preconditioning in myocytes. Yellon's group([324]) were the first to report, using the in vivo rabbit model, that the PKC inhibitor, chelerythrine, abolished the delayed protection using infarct size as the end-point. Subsequently, the same group demonstrated that a PKC agonist could mimic delayed ischaemic preconditioning.([325]) Translocation of the PKCε and PKCη isoforms have been implicated in mediating protection.([122;180;219;326-328]) PKC once activated is able to phosphorylate other kinase mediators.

1.3.5.3.b Tyrosine Kinase

The tyrosine kinase inhibitor, genistein has been demonstrated to abolish both the protection against stunning([329]) and infarction,([330]) induced by delayed preconditioning. Ping and colleagues([328]) subsequently established that src and lck tyrosine kinases are activated downstream of PKC and required for delayed cardio-protection.

1.3.5.3.c Mitogen Activated Protein Kinases (MAPK's)

An IPC protocol capable of inducing delayed cardio-protection via PKC can activate 3 major members of the MAPK family, Erk1/2, p38 and JNK MAP kinases.([219;327])

Punn and colleagues([307]) demonstrated that delayed preconditioning in myocytes was PKC but not Erk1/2-dependent. In contrast, Gross' group([331]) demonstrated that pharmacological activation of the δ-opioid receptor induced delayed cardio-protection via the p38 MAPK and Erk1/2. Furthermore, a recent study implicates ROS-induced Erk1/2 activation in

delayed preconditioning induced by pharmacological openers of the sarcolemmal and mitochondrial K_{ATP} channels.([332])

Yellon's group([333]) demonstrated that the delayed preconditioning effect induced by the adenosine A1-receptor agonist, CCPA, resulted in the activation of p38 MAPK via PKC and tyrosine kinase. Kukreja's group([334]) subsequently reported abrogation of delayed cardio-protection using SB203580, the p38 MAPK inhibitor. The same group later demonstrated that the pharmacological activation of p38 MAPK, using anisomycin induced delayed cardio-protection in the mouse via activation of NF-κB and iNOS, distal mediators/effectors of delayed preconditioning.([335])

1.3.5.3.d The PI3K-Akt Pathway

Recently, Yellon's group([336]) have demonstrated that the PI3K-Akt-p70S6K kinase cascade is required to mediate the delayed preconditioning-induced reduction in infarct size in the in vivo rabbit model. However, the downstream mechanisms involved in mediating the delayed preconditioning effect in this setting are currently unknown.

1.3.5.3.e Cardiac K_{ATP} Channels

Studies have implicated the sarcolemmal K_{ATP} and mitochondrial K_{ATP} channels as mediators of delayed preconditioning induced by ischaemia,([337]) heat stress,([338]) opioids,([339]) bradykinin,([322]) adenosine,([311;340]) or diazoxide (the putative mitochondrial K_{ATP} channel opener),([272;341]) The contribution of the different types of K_{ATP} channel to delayed preconditioning is unclear, but a recent study by Gross' group([339]) suggests that sarcolemmal K_{ATP} channel activation may act as a trigger and mitochondrial K_{ATP} channel activation may act as a mediator/effector of delayed preconditioning-induced protection. A recent study has suggested that the K_{ATP} channel openers, P-1075 and diazoxide, induced delayed cardio-protection by activating both the sarcolemmal and mitochondrial K_{ATP} channels, since the protection was abolished by 5-HD (the presumed mitochondrial K_{ATP} channel blocker) and HMR1098 (the sarcolemmal K_{ATP} channel blocker). In contrast, the K_{ATP} channel opener, BMS-191095, induced a delayed preconditioning effect which was not abolished by HMR1098, suggesting that it is more specific for the mitochondrial K_{ATP} channel, than either diazoxide or P-1075.([332]) The protection induced by all three K_{ATP} channel openers was demonstrated to be dependent on both ROS and Erk1/2.([332])

1.3.5.3.f Transcription Factors in Delayed Preconditioning

Recruitment of the kinases results in the activation of transcription factors, the most important one of which is nuclear factor-κB (NF-κB), which is expressed constitutively and is activated by preconditioning. It is known to regulate various distal mediators/effectors of delayed preconditioning (see section 1.3.5.4) such as iNOS, COX-2 and aldose reductase. Xuan and colleagues([342]) demonstrated that the activation of NF-κB occurred in response to a IPC stimulus or NO donors, and a non-specific inhibitor of NF-κB could abolish the delayed protective effect of preconditioning. Furthermore, inhibiting certain triggers/mediators such as NOS, PKC, tyrosine kinase, free radicals abolished the early activation of NF-κB, suggesting that the latter was a common downstream target of these triggers/mediators.([342]) Activation of NF-κB results from the phosphorylation of IκB (an inhibitory protein) by a distal MAP kinase called IKK. Maulik and colleagues([343]) demonstrated that IPC resulted in the phosphorylation of IκB by IKK, via a PKC-dependent mechanism. PKCε along with Erk1/2/JNK have been implicated in activating NF-κB in delayed preconditioning.([344])

There are probably other transcription factors involved in delayed preconditioning which are yet to be identified. One potential candidate is activating protein-1 (AP-1) which has been found to be activated in response to a brief ischaemia in rats.([344])

1.3.5.3.f The JAK-STAT Pathway

The Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway is a stress-responsive mechanism that transduces signals from the cell membrane to the nucleus, where gene expression is modulated (reviewed in reference ([224])). The JAK-STAT pathway is activated in response to an IPC stimulus and has been demonstrated to result in the transcriptional upregulation of iNOS([345]) and COX-2,([346]) known distal mediators/effectors of delayed preconditioning. Of the 4 JAK isoforms that have been described, JAK1 and JAK2, and of the 7 STAT isoforms that have been described, STAT1 and STAT3 have been implicated in delayed preconditioning.([224])

1.3.5.4 Distal Mediators and End-Effectors of Delayed Preconditioning

The temporal characteristics of delayed preconditioning obviate the requirement for the synthesis de novo of proteins which act as distal mediators/effectors, possibly under the regulatory control of transcription factors such as NF-κB and the JAK-STAT pathway. As such

these new proteins (for which the most evidence supports iNOS and COX-2) mediate their effect, 2-4 days following the original preconditioning stimulus. One study has reported that cycloheximide, a protein synthesis inhibitor, abolished delayed preconditioning in vivo.([347])

1.3.5.4.a Nitric Oxide Synthase

Vegh and colleagues([348]) were the first to postulate that delayed preconditioning against arrhythmias in dogs may require inducible nitric oxide synthase (iNOS) and/or cyclo-oxygenase (COX), following the finding that dexamethasone (which would inhibit NOS and COX) abolished the effect of delayed preconditioning. Subsequently, Bolli's group([349]) demonstrated an obligatory role for iNOS, as a distal mediator/effector in delayed preconditioning. L-NAME given just prior to the index ischaemic period, 24 hours after the IPC stimulus, abolished the delayed protective effect against both infarction([349]) and stunning.([350]) Selective iNOS inhibitors produced the same effect.([351]) Guo and colleagues([352]) demonstrated up-regulation of iNOS but not endothelial NOS (eNOS), and found that transgenic iNOS-knockout mice abrogated the delayed infarct-sparing effect.

Therefore, NO appears to play a dual role in delayed preconditioning, with NO generated from eNOS following the IPC stimulus acting as a trigger, and NO generated from iNOS 24 hours after the IPC stimulus acting as a distal mediator.([321]) Besides ischaemia, iNOS has been implicated in delayed preconditioning induced by diazoxide,([341]) but whether iNOS is required for CCPA-induced delayed preconditioning is unclear.([353;354])

1.3.5.4.b Cyclo-Oxygenase-2

Shinmura and colleagues([355]) demonstrated that COX-2 protein expression and myocardial levels of prostaglandin (PG) E_2 and /or PGI_2 were increased 24 hours following an IPC stimulus, and that inhibiting COX-2 24 hours after the IPC stimulus abolished the cardio-protective effects of delayed preconditioning. Subsequent studies demonstrated that COX-2 was downstream of iNOS in the preconditioned heart,([356]) and that the JAK-STAT pathway may mediate the up-regulation of COX-2.([346]) Recently, COX-2 was implicated in delayed preconditioning induced by heat stress.([357])

1.3.5.4.c Aldose Reductase

Aldose reductase (AR), is a member of the aldo-keto reductase family that has been shown to metabolise toxic aldehydes generated by lipid peroxidation. The protein expression of AR was demonstrated to be increased 24 hours after an IPC stimulus in conscious rabbits and the protective effect of delayed preconditioning was abolished if aldose reductase was inhibited.([358])

1.3.5.4.d Antioxidant Enzymes (MnSOD)

Studies have demonstrated an increase in the protein expression of manganese superoxide dismutase, 24 hours after a preconditioning stimulus using ischaemia,([359]) heat stress,([316]) exercise,([317]) or CCPA.([360]) In contrast, some studies found no increase in antioxidant enzymes 24 hours after the PC stimulus,([361]) so the role of antioxidants as a distal mediator is not clear.

1.3.5.4.d Heat Shock Proteins

Dillman's group([362]) reported that ischaemia induced the expression of mRNA for HSP72, and Knowlton and colleagues([363]) demonstrated an increase in HSP70 mRNA immediately following an IPC stimulus but also 24 hours after the stimulus. In Marber and colleagues' first description of delayed preconditioning, the elevation of HSP72 was noted 24 hours after the preconditioning stimulus.([44]) Subsequently, Yellon's group([333]) have implicated HSP27 in the delayed preconditioning effect induced by CCPA.

1.3.6 Other Forms of Preconditioning

1.3.6.1 Remote Preconditioning

Remote preconditioning describes a less recognised phenomenon, in which preconditioning of a region or an organ protects neighbouring tissues or remote organs, and was first described by Przyklenk and colleagues,([364]) in a study in which they demonstrated that a brief circumflex artery occlusion could precondition an area of myocardium served by the left anterior descending artery. Subsequently it has been demonstrated that ischaemia of many different organs such as kidney, mesentery, intestine and brain can protect the heart.([365-370]) The mechanisms involved in this phenomenon appear similar to that of classical preconditioning and involve adenosine, bradykinin, nitric oxide, PKC, inducible nitric oxide synthase, ([365-370]) Further

work is needed to elucidate the mechanisms involved and to evaluate the clinical potential of this form of preconditioning.

1.3.6.2 Calcium Preconditioning

Calcium-preconditioning (CPC) describes the phenomenon in which a transient small increase in intracellular [Ca^{2+}], triggers a preconditioning-like effect.([371-373]) Ashraf's group postulated that the transient rise in intracellular [Ca^{2+}] leads to a rapid adaptation of [Ca^{2+}] homeostasis and that the Ca^{2+} can activate different signalling pathways implicated in IPC-induced protection including PKC,([172;272]) and MAP kinases.([187;200]) It is likely, that CPC utilises the same signalling pathways as IPC, since the brief ischaemic episode of an IPC protocol would be expected to produce a transitory increase in intracellular [Ca^{2+}]. Studies have implicated PKC (a known mediator of IPC)([172;272]) as a mediator of CPC,([374;375]) but the role of the mitochondrial K_{ATP} channel in CPC, however, is unclear.([376;377]) Calcium-preconditioning has also been demonstrated in human myocardium and was shown to be mediated by PKC.([378])

1.3.7 Preconditioning the Human Myocardium

In order to translate the wealth of data available on myocardial preconditioning in animal hearts to the clinical arena of ischaemia-reperfusion injury (such as acute myocardial infarction, cardiac surgery, and percutaneous transluminal coronary angioplasty), an obligatory step is to demonstrate that the human myocardium can be protected by myocardial preconditioning, followed by the evaluation of these preconditioning strategies in large well-conducted randomised placebo-controlled clinical trials.

1.3.7.1 In Vitro Studies

Ikonomidis and colleagues([379]) were the first to demonstrate that hypoxic preconditioning could protect human ventricular myocytes against hypoxia-reoxygenation injury, as evidenced by reduced trypan blue uptake and attenuated LDH release. The same group went on to implicate a role for adenosine and PKC using the same model.([380]) Arstall and colleagues([310]) subsequently demonstrated that a period of hypoxia could elicit a delayed preconditioning effect in foetal human myocytes.

Yellon's group([381]) have demonstrated that human adult atrial trabeculae harvested at the time of cardiac surgery can be hypoxically preconditioned, using the recovery of baseline contractile function as the end-point. Using this model, they have demonstrated that adenosine A1 and A3([382]) and δ-opioid([383]) activation can act as triggers of preconditioning, and have implicated PKC and the mitochondrial K_{ATP} channel as mediators of preconditioning.([384;385]) Preconditioning using this model has been confirmed by another laboratory.([386;387])

Galinanes group have demonstrated hypoxic preconditioning, in slices of human adult atrial appendage harvested at the time of cardiac surgery, using the release of creatine kinase as an indicator of necrosis.([312;388-392])

1.3.7.2 In Vivo Studies

It has been suggested that ***warm-up angina*** (which describes the phenomenon in which a patient can exercise for longer without symptoms, following an episode of angina) may be due to a preconditioning-like effect,([393-398]) rather than due to the recruitment of collateral vessels as previously thought. However, certain components normally associated with myocardial preconditioning such as adenosine([399]) and K_{ATP} channels([400-402]) do not appear to be implicated in the phenomenon of warm-up angina, suggesting that the mechanism may not actually be preconditioning.

From retrospective analyses of patients who have had an acute myocardial infarction, the presence of ***pre-infarction angina*** has been associated with an attenuated release of creatine kinase, improved left ventricular function, reduced ventricular arrhythmias, reduced incidence of congestive cardiac failure, and reduced mortality,([403-410]) and even a smaller infarct size.([411]) Other studies however, have reported no benefit with angina, antecedent to primary PTCA for myocardial infarction.([412]) Whether this apparent protective effect is due to myocardial preconditioning is unknown,([413]) but studies suggest that pre-infarction angina is only protective if it occurs within 24-72 hours of the myocardial infarction,([403;407;411]) which interestingly corresponds to the time-frame of delayed preconditioning-induced protection.

A surrogate model for myocardial preconditioning has been demonstrated in the setting of ***percutaneous transluminal coronary angioplasty*** (PTCA). Studies have demonstrated that an intracoronary inflation lasting longer than 60-90 seconds, can improve indicators of myocardial ischaemia for subsequent inflations, independent of collateral vessel recruitment. These indices of myocardial ischaemia include chest pain, abnormalities of left ventricular

regional wall motion, S-T segment elevation, ventricular ectopic activity, lactate release and CKMB release.([414-418]) Interestingly, the mechanisms implicated in this protective effect are those known to mediate classical preconditioning and include K_{ATP} channels,([419;420]) adenosine,([421-423]) opioids,([424]) and bradykinin.([425])

With respect to delayed preconditioning, Bolli's group([426]) demonstrated that an intravenous infusion of nitroglycerin, 24 hours prior to the PTCA procedure, attenuated the symptoms of chest pain, attenuated S-T segment elevation and improved regional ventricular wall motion compared to control patients. Marber's group([427]) have recently demonstrated that an exercise tolerance test (ETT) can confer a myocardial adaptive effect 24 hours later, with an improved exercise tolerance and less ventricular ectopics during an ETT and attenuated S-T segment elevation at the time of PTCA.

Another surrogate model for myocardial preconditioning of the human myocardium is at the time of ***cardiac surgery***, during which time the heart is subject to sustained periods of global ischaemia induced by aortic cross-clamping, for the insertion of coronary artery bypass grafts. Yellon's group and others have demonstrated that intermittent cross-clamping of the aorta prior to the sustained episode of global ischaemia protected the human hearts as evidenced by the preservation of ATP levels,([428]) and attenuated troponin T release.([429-431]) However, applying this preconditioning protocol when using either hypothermia or cardioplegic arrest or a combination of both to protect the heart has produced mixed results, with some studies reporting a beneficial effect,([431-433]) but others not.([434;435]) Pharmacological preconditioning using adenosine produced a negative result,([436]) and in another study ischaemic preconditioning was more beneficial that an adenosine A1 agonist.([430])

Preconditioning in cardiac surgery has met with resistance because intermittent cross-clamping may prolong surgery by 15-30 minutes and represents an embolic risk, and has not been examined in terms of mortality and morbidity. In addition, ischaemic preconditioning in cardiac surgery may offer no more protection than that obtained by the drugs used for pre-medication (opioids)([383]) and anaesthesia (isoflurane),([436]) cardiopulmonary bypass([33]), all interventions which in themselves may protect the heart.

Therefore preconditioning in cardiac surgery will only become an important and widespread technique if a pharmacological alternative to intermittent cross-clamping can be demonstrated to be an effective preconditioning strategy in large-scale clinical trials.

1.3.7.3 Preconditioning in the Clinical Arena

The evidence suggests that the human myocardium can be preconditioned but in the clinical settings of ischaemia-reperfusion injury such as acute myocardial infarction, cardiac surgery, and PTCA, preconditioning by an episode of antecedent ischaemia is not clinically feasible, and pharmacological agents which have been demonstrated in animal models to precondition the myocardium need to be examined in large scale clinical trials. Another issue is the patient group which is likely to benefit from such an intervention and the logistics of applying an effective preconditioning strategy, given the unpredictable onset of an acute myocardial infarction, the patients likely to benefit from a preconditioning strategy, which by definition needs to be instituted prior to the ischaemic insult, will be high-risk unstable coronary artery disease patients and those undergoing cardiac surgery. Otherwise, the putative preconditioning agent would have to be administered to patients with chronic stable coronary artery disease which would raise the issue of tachyphylaxis, although a recent study by Yellon's group([437]) suggests that the intermittent treatment of a known preconditioning agent, an A1 adenosine receptor agonist may be able to maintain a preconditioned state over several days.

Nicorandil, a putative mitochondrial K_{ATP} channel opener has been examined as a potential preconditioning agent in the clinical setting in patients with unstable coronary artery disease and was demonstrated to attenuate myocardial ischaemia and ischaemia-induced arrhythmias.([438]) The Impact Of Nicorandil on Angina (IONA) study examined nicorandil in patients with chronic stable coronary artery disease and demonstrated a small but significant reduction in major coronary events.([439]) However, in both these studies the vasodilatory property of this drug cannot be discounted from the apparent protective effect.

This section has reviewed the phenomenon of myocardial preconditioning, a mechanism of myocardial protection which needs to be instituted before the index ischaemic episode to be effective. The next section reviews an alternate treatment strategy for myocardial protection which allows intervention at the time of reperfusion, following the index ischaemic episode.

1.4 Myocardial Protection by Intervening at Reperfusion

1.4.1 Targeting Lethal Reperfusion Injury

Following an acute myocardial infarction, re-establishing coronary blood flow with the rapid use of reperfusion strategies such as thrombolysis or primary angioplasty, is a pre-requisite to myocardial salvage. However, the restoration of coronary blood flow to the ischaemic myocardium carries with it an inherent risk, in that paradoxically, the process of reperfusion can itself result in myocyte death-a phenomenon termed lethal reperfusion injury.([6]) The non-lethal forms of reperfusion injury which comprise myocardial stunning and reperfusion arrhythmias will not be dealt with in this section.

Lethal reperfusion injury is defined as injury caused by restoration of blood flow after an ischaemic episode leading to death of cells that were only reversibly injured during that preceding ischaemic episode.([440]) The existence of lethal reperfusion injury as an entity separate from ischaemic injury is controversial and has been debated over the years, with some commentators suggesting that reperfusion only exacerbates the cellular injury sustained during the ischaemic period.([441])

However, other studies have demonstrated that reperfusion can exacerbate the necrotic component of cell death as evidenced by an extension in infarct size, following a fixed period of ischaemia.([442;443]) Other studies, on the other hand, indicate that the oxidative stress and abrupt metabolic changes that accompany reperfusion can initiate cellular injury in the absence of ischaemia.([444;445])

In order to prove the existence of lethal reperfusion injury as an independent cause of cell death it is necessary to demonstrate that myocytes which are viable immediately prior to reperfusion, are irreversibly injured by the act of reperfusion. Because the progress of necrosis following an episode of ischaemia-reperfusion is difficult to follow accurately, the most convincing means of demonstrating the existence of lethal reperfusion injury has been to show that myocyte death can be modified by interventions administered *at the time of reperfusion.* The definitive evidence of lethal reperfusion injury requires a reproducible, controlled study in which an intervention given at the time of reperfusion (and subsequently withdrawn) results in a significant and sustained improvement in infarct size, cardiac function and cardiac metabolism, when assessed 4 or more days after a period of ischaemia.([446]) Of course many studies do not

conform to all these rigorous criteria, which may in part explain the disparity and confusion in findings from studies investigating the contribution of lethal reperfusion injury. This section explores interventions that have been demonstrated to protect the heart against lethal reperfusion injury, by modifying factors that have been implicated as critical determinants of lethal reperfusion injury, such as reactive oxygen species, calcium overload, and pH changes. Finally, the role of the survival kinase cascades PI3K-Akt and MEK1/2-Erk1/2, that have been demonstrated to mediate protection against lethal reperfusion injury are examined.

1.4.1.1 Reactive Oxygen Species as Mediators of Lethal Reperfusion Injury

Free radicals such as superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), are produced on reperfusion of ischaemic myocardium and represent a major cause of lethal reperfusion injury.([79;276;277]) The source of the free radicals generated during the reperfusion phase include xanthine oxidase,([447;448]) and the mitochondrial respiratory chain.([449]) The catalytic breakdown products ADP and AMP which result from the ATP hydrolysis that takes place during ischaemia, are converted to hypoxanthine, which is oxidised to produce oxygen free radicals.([447]) The reoxygenation of a reduced mitochondrial respiratory chain has been demonstrated to be a major source of free radicals in the transition from ischaemia to reperfusion.([450]) In particular, ubiquinone is believed to be the site of ROS generation.([449])

Free radicals cause damage to cell membrane phospholipids and cellular proteins resulting in metabolic and structural changes. Free radicals induce lipid peroxidation, generating lipid peroxides which can induce damage to both cellular and membranous proteins.([451]) The oxidation of membranous thiol groups can induce the opening of the mitochondrial permeability transition pore (mPTP), which leads to irreversible cell injury (see section 1.5.2.1.b).([452;453]) Therefore, the pro-injurious effects associated with the burst of free radicals which are produced at the time of reperfusion offer a target for protection against lethal reperfusion injury.

In this regard, in 1984, Jolly and colleagues,([454]) were the first to demonstrate, that scavenging free radicals at the time of reperfusion protected the myocardium against lethal reperfusion injury, as evidenced by a reduction in infarct size. Using a canine model subjected to 90 minutes of left circumflex coronary artery occlusion, they demonstrated that infusing superoxide dismutase (SOD) and catalase into the left atrium 15 minutes before reperfusion, reduced infarct size by 50%, although free radical scavengers given 40 minutes after the onset of reperfusion were ineffective, underlining the importance of intervening during the early

reperfusion phase.([454]) Other studies however, have failed to show any infarct limitation using the same model of ischaemia-reperfusion.([455-457]) Variations in collateral flow,([458]) and the techniques used for measuring infarct size,([459]) have been suggested explanations for the conflicting results. Studies which fail to assess collateral flow (pertinent in the canine heart where collateral flow can vary from 5% to 80%) often give false-negative results.([458]) Measuring infarct size by tetrazolium staining can detect necrotic tissue not detected by normal histological techniques.([459]) Studies using transgenic mice over-expression SOD endured 50% smaller infarct sizes compared to non-transgenic control, following ischaemia-reperfusion.([460])

1.4.1.2 Calcium Overload

In the first few minutes of reperfusion there is an abrupt accumulation of cytosolic calcium which in turn activates phospholipases, proteases and nucleases. In 1972, Shen & Jennings,([21;22]) using the canine heart, were the first to demonstrate the accumulation of calcium overload on reperfusion of ischaemic myocardium. In addition, the rise in cytosolic calcium enters the mitochondria via the Ca^{2+}-uniporter driven by the repolarised mitochondrial membrane potential, and can trigger the opening of the mPTP, which can mediate cell death.([452])

Therefore, preventing the calcium overload that occurs at the time of reperfusion is another target for protecting the heart against lethal reperfusion injury. In this regard, studies have demonstrated that calcium antagonists administered at the time of reperfusion can protect against lethal reperfusion injury.([461-463]) Using the swine model of ischaemia-reperfusion, Herzog and colleagues([463]) found that diltiazem given for the first 12 minutes of reperfusion reduced infarct size. In the same model, Klein and colleagues([461]) demonstrated a reduction in infarct size with the intracoronary infusion of either nifedipine or verapamil, and Hatori and colleagues([462]) found that coronary venous retroperfusion with felodipine reduced infarct size.

The influx of calcium into mitochondria at the time of reperfusion can be targeted directly, by using ruthenium red, an inhibitor of the Ca^{2+}-uniporter. Several studies have demonstrated protection against lethal reperfusion/reoxygenation injury with this agent, using the in vivo porcine heart([464]), and the isolated perfused rat heart,([465]), Miyamae and colleagues([466]) demonstrated directly in the isolated perfused rat heart that ruthenium red inhibited the increase in mitochondrial Ca^{2+} at the time of reperfusion, and improved post-ischaemic recovery in contractile function.

1.4.1.3 The pH Paradox and Na^+/H^+ Exchanger Inhibitors

The intracellular acidification (pH<7.0) which occurs during ischaemia has been demonstrated to be protective.([467;468]) Therefore its abrupt reversal at the time of reperfusion to neutral pH due to wash-out of lactic acid and the activation of the Na^+/H^+ exchanger, contributes to the cell death from lethal reperfusion injury-termed the pH paradox.([469]) Furthermore, the action of the Na^+/H^+ exchanger to extrude H^+ results in the activation of the Na^+/Ca^{2+} exchanger, which in turn causes calcium overload at the expense of Na^+ extrusion. Intracellular acidosis is believed to protect the myocardium by inactivating phospholipases and proteases as well as inhibiting mPTP opening.([469]) Lemaster's group([470]) have demonstrated that the reoxygenation of hypoxic myocytes with acidic buffer can protect the cells against lethal reoxygenation injury by inhibiting mPTP opening.

Maintaining a low intracellular pH during the first few minutes of reperfusion may therefore be a target for protection against lethal reperfusion injury. In this regard, administering a Na^+/H^+ exchanger inhibitor, such as HOE 694,([471]) cariporide (HOE 642),([472]) or EMD-85131,([473]) at the time of reperfusion has been demonstrated to protect against lethal reperfusion injury. However, there are studies that report no protective effective associated with Na^+/H^+ exchanger inhibitors against lethal reperfusion injury,([474-476]) which supports the general notion that these class of drugs are most effective when administered prior to or after the onset of the index ischaemic period.([477])

1.4.1.4 Nitric Oxide

In the first few minutes of reperfusion, the endothelial production of nitric oxide (NO) is impaired in response to the inactivation of NO by superoxide, resulting in endothelial dysfunction.([478]) On this basis, the restoration of NO at the time of reperfusion offers a potential target for protecting against lethal reperfusion injury. In this regard, the presence of exogenous nitric oxide, in the form of NO dissolved in aqueous medium or NO donors can reduce infarct size in various models of ischaemia-reperfusion.([479-481]) Transgenic mice lacking the gene for endothelial nitric oxide synthase (eNOS) sustain larger infarcts when subjected to ischaemia-reperfusion.([28;482]) Cardio-protective mechanisms of NO at reperfusion include: attenuation of neutrophil infiltration,([483]) the inactivation of superoxide radicals,([484]) or the improved coronary blood flow secondary to NO-induced vasodilation.([485])

However, there are studies that report the contrary, that NO is detrimental at reperfusion,([486;487]) and one study reported that functional recovery following ischaemia-reperfusion was improved in eNOS-knockout mice when compared to non-transgenic controls([488]) The dichotomous role of NO may be due to the concentration of NO and the source of NO, whether it is produced by eNOS or inducible nitric oxide synthase (iNOS). With regard to the former, this may have implications on mPTP opening at the time of reperfusion, with small quantities of NO inhibiting mPTP opening and larger amounts inducing mPTP opening.([489]) In this regard, Lemasters and colleagues have demonstrated that the NO donor SNAP administered to rat myocytes at the time of reoxygenation can protect against lethal reoxygenation injury by inhibiting mPTP opening (unpublished data).

1.4.1.5 Adenosine

In addition to acting as a trigger for classical preconditioning, adenosine has been demonstrated to mediate protection against lethal reperfusion injury, although there are studies reporting the contrary. The intracoronary infusion of adenosine in a canine model of myocardial infarction, given at the onset of reperfusion reduced infarct size.([490]) Agonists for the adenosine A1, A2, and A3 receptor sub-types have been demonstrated to protect against lethal reperfusion injury, when given at the time of reperfusion.([491-493]) The adenosine A1/A2a receptor agonist, AMP 579, has been observed in various animal models of ischaemia-reperfusion to protect against lethal reperfusion injury, when administered at the time of reperfusion.([494-497]) However, there have also been several studies reporting no protection with adenosine given at the time of reperfusion.([498;499])

The mechanism of adenosine-induced protection at the time of reperfusion has been attributed to attenuation of neutrophil infiltration, reduced superoxide generation and attenuated endothelial dysfunction,([493]) and in the case of the adenosine agonist, AMP 579, the MEK1/2-Erk1/2 pathway([494]) has been implicated.

1.4.1.6 The Renin-Angiotensin System

Angiotensin II type 1 receptor blockers such as candesartan([500]) and losartan have been demonstrated to protect against lethal reperfusion injury by potentiating bradykinin stimulation at the bradykinin B2 receptors([501]) and releasing nitric oxide.([502])

1.4.2 The Contribution of Apoptosis to Lethal Reperfusion Injury

Apoptotic cell death in the rat heart has been demonstrated to be induced by a prolonged episode of ischaemia alone, in the absence of reperfusion.([503;504]) Some studies have suggested that reperfusion accelerates the apoptotic death process initiated during ischaemia.([503;505-507]) In contrast, several studies suggest that the apoptotic component of cell death is triggered at the time of reperfusion, and does not manifest during the ischaemic period.([508]) Therefore, the evidence suggests that the apoptotic component of cell death is either triggered or accelerated during the reperfusion phase. The fact that apoptosis is an energy-dependent process and ATP levels are depleted during ischaemia and replenished on reperfusion, may explain why the apoptotic component of cell death is associated with reperfusion.([509])

The *relationship* between apoptotic and necrotic cell death in the setting of ischemia-reperfusion injury is also unresolved, with some commentators suggesting that there may be considerable overlap in terms of early signalling events between these two pathways, an observation that may be useful in terms of developing therapeutic targets for clinical use. Zhao and colleagues([510]) have characterised, using a canine model of ischaemia-reperfusion injury, the contribution of necrotic and apoptotic cell death. They demonstrated that these two forms of cell death occur simultaneously during the reperfusion phase, with necrotic cell death peaking after 24 hours reperfusion, and apoptotic cell death increasing up to 72 hours of reperfusion.([510]) Other studies have demonstrated that the pharmacological inhibition of the apoptotic signalling cascade during the reperfusion phase, is able to attenuate both the apoptotic and necrotic components of cell death,([511-514]) suggesting that the apoptotic death process can evolve into necrotic cell death. As well as the apoptotic component of cell death contributing to the extension of infarct size during reperfusion, a study by Zhao and colleagues, ([514]) demonstrated that pharmacologically inhibiting the reperfusion-induced apoptotic component of cell death also resulted in improved contractile function of ischaemic canine hearts.

However, although it is fair to state that the majority of evidence supports the role of apoptosis in ischaemia-reperfusion injury, because of unresolved issues surrounding the contribution of apoptosis to the pathophysiology of ischaemia-reperfusion injury, several authors still question the significance of apoptosis in this setting.([515]) For example, methodological issues concerning the detection of apoptosis in the heart, were questioned in a study by Ohno

and colleagues,([516;517]) in which immunogold electron microscopy and in situ nick end labelling, revealed that coronary artery occlusion in the rabbit resulted in detection of necrotic and not apoptotic cell death, and Taimor and colleagues([518]) could only demonstrate the induction of necrosis but not apoptosis in isolated rat myocytes subjected to hypoxia-reoxygenation.

These studies suggest that targeting the reperfusion-induced apoptotic component of cell death can impact on both the apoptotic and necrotic components of cell death, the consequences of which are a reduction in infarct size and improved contractile function. Yellon's laboratory([519]) have been investigating the role of specific pro-survival signalling kinase cascades at the time of reperfusion, which protect the heart against lethal reperfusion injury by targeting the apoptotic component of cell death.

1.4.3 The Reperfusion Injury Salvage Kinase Pathway: The RISK-Pathway

Yellon's laboratory([519]) formulated and tested the hypothesis that the pharmacological activation of the signalling kinase pathways, phosphatidylinositol-3-OH kinase (PI3K)-Akt, and p42/p44 extra-cellular signal-regulated kinases (Erk 1/2), at the time of reperfusion protects the myocardium against lethal reperfusion injury. Activation of these kinase cascades (which have been termed the Reperfusion Injury Salvage Kinase Pathway [The RISK-Pathway]),([520]) protects the heart by recruiting several anti-apoptotic pathways of cellular survival (see figure 1.5).

1.4.3.1 Protecting the Heart Against Lethal Reperfusion Injury by Activating the (RISK)-Pathway at Time of Reperfusion

A number of growth factors and other agents have been shown to induce cardio-protection in the setting of ischaemia-reperfusion injury. This section will focus on those that have been shown to protect the heart when given *during the early reperfusion phase*, and whose mechanism of protection has been linked to activation of these pro-survival kinase cascades. Table 1.1, provides a summary of these agents which have been demonstrated to protect against lethal reperfusion injury, by activating either the PI3K-Akt and/or Raf1-MEK1/2-Erk1/2 kinase cascades, when given at the time of reperfusion.

Table 1.1: Summary of Agents Which Protect at Reperfusion by Activating Akt/Erk1/2. These agents protect at the time of reperfusion via activating the: G-protein coupled receptor (GPCR), NO receptor (NR), glycoprotein 130 (gp130) receptor, the tyrosine kinase (TK) receptor or serine threonine kinase receptor, which then activates either the Akt or Erk1/2 of the Reperfusion Injury Salvage Kinase (RISK) pathway.

Protective Agent	Receptor	RISK-Pathway		Model	Reference
		Akt	Erk1/2		
AMP579	GPCR		✓	In vivo rabbit model	Kis 2004([521])
Atorvastatin	NR	✓	✓	Isolated mouse heart	Bell 2003([522]) Efthymiou([523])
Bradykinin	GPCR	✓		Isolated mouse heart	Bell 2003([524])
			✓	In vivo rabbit model	Downey (unpublished)
CT-1	gp130		✓	Isolated rat heart	Liao 2002([525])
		✓	✓	Adult rat myocytes	Brar 2001([526;527])
FGF	TK		✓	In vivo rat heart	Cuevas 1997,1999([528;529])
Insulin	TK	✓		Isolated rat heart	Jonassen 2001([530])
		✓		In vivo rat heart	Gao 2002([531])
IGF-1	TK	✓		Isolated rat heart	Otani 2000([532])
		✓		In vivo mouse heart	Yamashita 2001([533])
TGF-β1	Ser Thr Kinase		✓	Isolated rat heart	Baxter 2001([534])
			✓	Adult rat myocytes	Baxter 2001([534])
Urocortin	TK		✓	In vivo rat heart	Schulman 2002([535])
			✓	Isolated rat heart	Schulman 2002([535])Brar 2000([536])
		✓		Adult rat myocytes	Brar 2000,2002([536;537])

1.4.3.1.a Insulin

Jonassen and colleagues([538]) demonstrated using an in vivo rat heart model of ischaemia-reperfusion injury, a reduction in infarct size associated with the administration of glucose-insulin-potassium (GIK) at the time of reperfusion. In studies using rat cardiomyocytes subjected to hypoxia, Yellon's laboratory([539]) demonstrated that insulin given, at the time of reoxygenation, attenuated the apoptotic and necrotic components of cell death. In order to determine the mechanism of insulin-induced protection at the time of reperfusion, Yellon's group([530]) demonstrated using the isolated perfused rat heart, that administering insulin at the time of reperfusion activated the PI3K-Akt-p70S6K-BAD pathway, and that inhibitors of this pathway such as wortmannin (the PI3K inhibitor), and rapamycin (the p70S6K inhibitor), abrogated insulin-induced protection. Administering insulin at the time of reperfusion activated this kinase cascade.([530]) The early perfusion of insulin was essential for protection, as delaying its administration to 15 minutes after the onset of reperfusion, was not associated with protection, suggesting that the protection via the PI3-Akt kinase cascade was mediated in the first few minutes of reperfusion.([530]) Using an in vivo rat heart model of ischaemia-reperfusion injury, Gao and colleagues([531]) have also implicated eNOS, another downstream target of Akt phosphorylation, in insulin-mediated cardio-protection at reperfusion.

1.4.3.1.b Insulin-Like Growth Factor-1 (IGF-1)

IGF-1 is a serum factor implicated in cellular survival and growth that has been shown to reduce apoptosis in a wide variety of cells in response to a diverse array of stimuli. IGF-1 has been demonstrated to protect the heart against ischaemia-reperfusion injury, by attenuating both apoptotic and necrotic cell death.([533;540]) in a manner that is dependent on the PI3K-Akt and Erk 1/2 signalling cascades.([541;542])

In the isolated perfused rat heart, the administration of IGF-1 at reperfusion protected the heart against lethal reperfusion-injury in a wortmannin-sensitive manner, suggesting the involvement of the PI3K-Akt pathway.([532]) Studies in transgenic mice over-expressing IGF-1, displayed protection against ischaemia-reperfusion injury, and were found to have a higher basal activation of Akt, and interestingly, at reperfusion the level of Akt activation was amplified even further.([533]) Downstream of the these kinase cascades, Bad,([543]) Bax, caspase 3,([544]) and p70S6K have been implicated in IGF-1 induced cellular protection.

1.4.3.1.c Transforming Growth Factor-β1

Transforming Growth Factor-β1 (TGF-β1) is a cytokine that regulates cell growth and differentiation and modulates apoptosis in many cell types. The cardio-protective properties of TGF-β1 were first investigated in the early 1990's by Lefer and colleagues who demonstrated protection against ischaemia-reperfusion injury in the rat heart ex vivo and in vivo.([478])

Yellon's group([534]) were the first to study the effect of TGF-β1 when given at the point of reoxygenation/reperfusion in rat myocytes and in the isolated perfused rat heart, respectively. In this study, TGF-β1 given during the reoxygenation phase following an episode of lethal hypoxia, was shown to be protective, demonstrated by attenuated trypan blue uptake and a reduction in the apoptotic component of cell death (assessed by a reduction in TUNEL and annexin V-positive cells) and in the isolated perfused rat heart, treatment with TGF-β1 for the first 15 minutes of reperfusion, following an episode of lethal ischaemia, was associated with a significant reduction in infarct size. In both these cases TGF-β1-induced protection was demonstrated to be dependent on the Erk 1/2 cascade.([534])

1.4.3.1.d Cardiotrophin-1

Cardiotrophin-1 (CT-1) is a member of the interleukin 6 family of cytokines, which was originally isolated for its ability to induce a hypertrophic response in isolated cardio-myocytes,([545]) by signalling through the gp130 trans-membrane protein.([546]) CT-1 has since been shown to be cardio-protective, exerting an anti-apoptotic effect in response to serum withdrawal in cardiac myocytes via an Erk 1/2-dependent pathway.([547]) Using the isolated rat myocyte and the isolated perfused rat heart models, Yellon's group demonstrated that, when given at point of reoxygenation/reperfusion, CT-1 induced protection against lethal reperfusion injury via activation of the MEK1/2-Erk 1/2 ([525;526]) and the PI3K-Akt pathways.([527])

1.4.3.1.e Urocortin

Urocortin, a peptide related to hypothalamic corticotrophin releasing factor, is released by myocytes in response to stressful stimuli such as ischaemia.([548]) Using neonatal rat myocytes, Brar and colleagues ([536]) demonstrated that after a prolonged episode of hypoxia, the presence of urocortin, at the time of reoxygenation, prevented cell death, by an anti-apoptotic action (represented as a reduction in annexin V and TUNEL staining). Yellon's laboratory went on to

demonstrate, in the isolated perfused rat heart and in-vivo rat heart subjected to prolonged ischaemia, that urocortin given at the time of reperfusion reduces infarct size by activating the MEK1/2-Erk1/2 kinase cascades.([535;536]) Brar and colleagues also demonstrated in neonatal rat myocytes that urocortin induced the expression of HSP90 in a MEK1/2-dependent manner.([549])

In a further study Brar and colleagues demonstrated that urocortin-mediated protection against hypoxia-reoxygenation injury was abrogated in rat myocytes treated with the specific PI3K-inhibitors, wortmannin or LY294002, implicating the PI3K-Akt kinase cascade in urocortin-induced cardio-protection.([537]) In addition, urocortin-induced protection in the same setting was abrogated in neonatal rat myocytes possessing a dominant negative mutation of PI3K-Akt.([537])

1.4.3.1.f Fibroblast Growth Factor

Fibroblast growth factor-2 (also known as basic fibroblast growth factor, FGF) is a polypeptide growth factor, which has been shown to modulate cell proliferation, survival and apoptosis.([550]) FGF has been demonstrated to protect against lethal reperfusion injury, when administered during the reperfusion phase in an in vivo rat heart model of myocardial infarction by increasing iNOS,([529;551]) and has been associated with attenuation of apoptotic cell death.([528]) Studies have implicated PKC and the Erk1/2 pathway in FGF-mediated cardio-protection.([552;553]) Jiang and colleagues([554]) demonstrated cardio-protection in the isolated perfused rat heart when FGF was given during the first 12 minutes of reperfusion, following 30 min of global ischaemia, and protection was shown to be PKC-dependent. The fact that FGF-mediated cardio-protection has been shown to be dependent on PKC and possibly K_{ATP} channels,([555]) suggests that FGF may protect via a similar mechanism to ischaemic preconditioning.

1.4.3.1.g Other Cardio-Protective Growth Factors

Several other growth factors including vascular endothelial growth factor([556]) and hepatic growth factor([557;558]) have been shown to cardio-protect against ischaemia-reperfusion injury but have not been examined at the time of reperfusion. Hepatic growth factor was demonstrated to induce activation of Erk 1/2 kinase, but the contribution of this kinase to cellular protection was not examined in this study.([558])

Other growth factors such as epidermal growth factor,([559]) nerve growth factor([560]) and platelet-derived growth factor,([560]) have been shown to be protective but have not yet been investigated in cardiac tissue. One can postulate that if these growth factors activate the pro-

survival kinases cascades of the RISK-pathway, in myocardial tissue, one might expect them to also protect the heart against lethal reperfusion injury.

1.4.3.1.h HMG-CoA Reductase Inhibitors

The hydroxyl-3-methylglutaryl (HMG)-co-enzyme A (CoA) reductase inhibitors or "statins" have been shown to be cardio-protective in large-scale primary ([561]) and secondary prevention clinical studies.([562]) In addition to its cholesterol-lowering effect, this class of drugs has been associated with many pleiotropic effects, many of which mediate their cardio-protective effect.([563]) HMG-CoA reductase inhibitors have also been shown to up-regulate the PI3K-Akt kinase cascade, in endothelial cell lines.([564])

Yellon's group,([522]) using the isolated perfused mouse heart model, demonstrated that the HMG-CoA reductase inhibitor, atorvastatin, administered during the early reperfusion phase, limited infarct size via recruitment of the PI3K-Akt kinase pathway. Furthermore, the downstream activation of eNOS was also implicated in atorvastatin-induced cardio-protection at reperfusion, based on the finding that atorvastatin provided no cardio-protection in mice with a targeted deletion of the eNOS gene.([522])

Recently, this group have also demonstrated that atorvastatin administered at the time of reperfusion in the isolated perfused mouse heart, protects against lethal reperfusion injury as evidenced by a reduction infarct size, via the activation of the MEK1/2-Erk1/2 kinase cascade.([523])

1.4.3.1.i Bradykinin

Treatment with angiotensin-converting enzyme inhibitors (ACE-I) have been linked to cardio-protection in the setting of ischaemia-reperfusion injury.([565]) Studies have demonstrated that ACE-I induced cardio-protection is mediated by bradykinin (acting at the B2 receptor) and is dependent on nitric oxide.([566]) Interestingly, it has been shown that Gq-protein receptors, such as the bradykinin B2 receptor signal through the PI3K pathway in the guinea pig heart.([567])

Based upon this, Yellon's group([524]) demonstrated for the first time a link between G protein coupled receptor activation at reperfusion, using bradykinin, and cardio-protection via recruitment of the PI3K-Akt pathway. Using the isolated perfused mouse heart model, they found that bradykinin administered during the first few minutes of reperfusion, limited infarct size via recruitment of the PI3K-Akt kinase pathway.([524]) Furthermore, bradykinin-induced cardio-

protection was shown to be eNOS dependent, as bradykinin provided no cardio-protection in transgenic eNOS knockout mice.([524])

Interestingly, a recent study in bovine aortic endothelial cells has shown that bradykinin can activate Erk 1/2 and eNOS activation, independent of the PI3-Akt pathway.([568]) This suggests that the Erk 1/2 kinase pathway may also contribute to bradykinin-mediated cardio-protection at reperfusion. This is supported by the recent findings of Downey and colleagues who found that administering bradykinin or NECA at the time of reperfusion protected the in vivo rabbit heart against lethal reperfusion injury via the MEK1/2-Erk1/2 cascade (unpublished data).

1.4.3.1.j Adenosine agonists

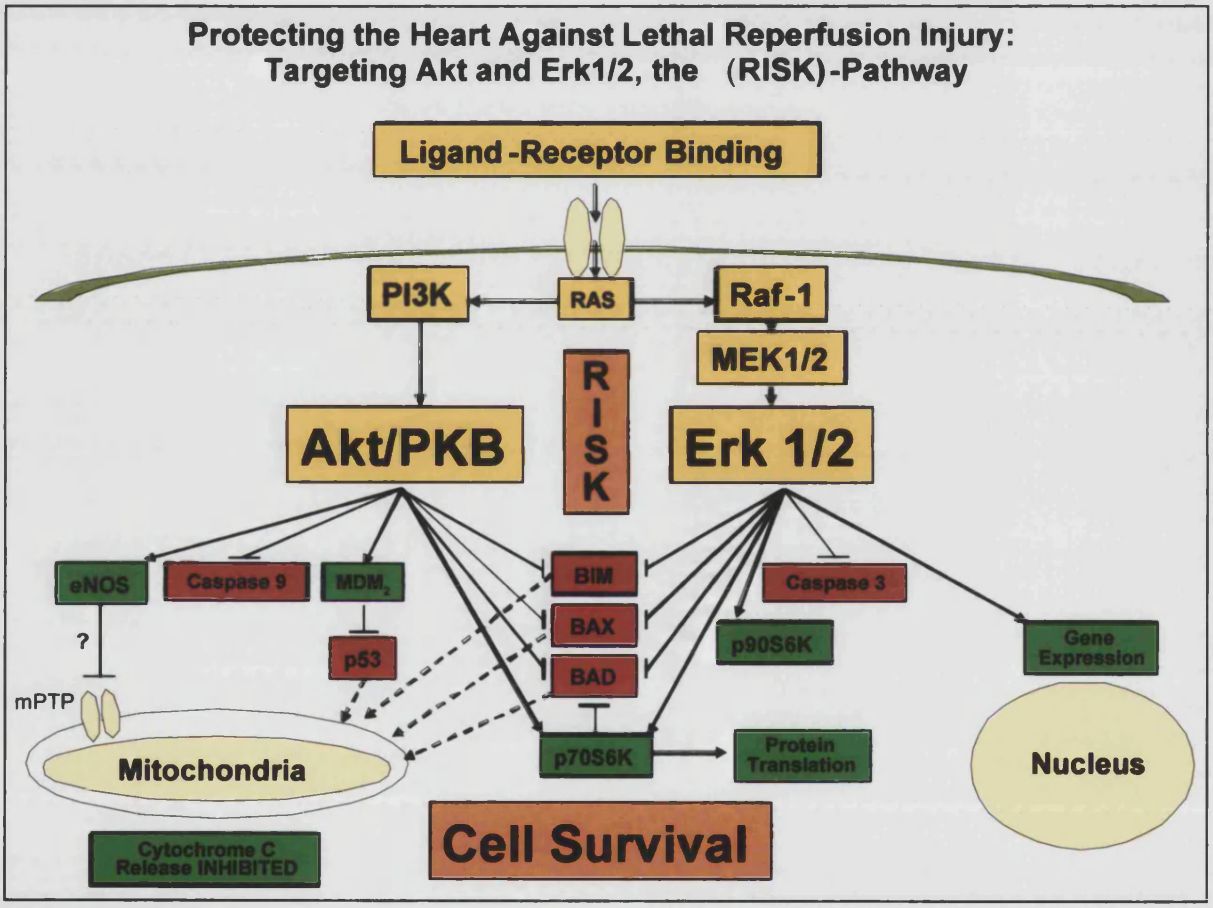
Xu and colleagues demonstrated that AMP579 (an adenosine A1/A2a receptor agonist) given during the reperfusion phase limited infarct size using the in vivo rabbit heart model of ischaemia-reperfusion injury.([496]) Interestingly, activation of the adenosine A1, A2 and A3 receptor has been associated with phosphorylation of the Erk 1/2 kinase cascade in Chinese Hamster ovary cells.([569]) Using the in vivo rabbit heart model of ischaemia-reperfusion injury, Yellon's group found that AMP579-induced protection at reperfusion, was abrogated in the presence of PD 98059 (the MEK 1/2 inhibitor).([521])

1.4.3.2 The PI3K-Akt Kinase Signalling Cascade

The activation of the pro-survival PI3K-Akt and MEK 1/2-Erk 1/2 cascades at the time of reperfusion, by ligands to growth factor or G-protein coupled receptors, protects the heart against lethal reperfusion injury. The protective mechanisms induced by these kinase cascades are mediated by the diverse array of substrates which these kinases phosphorylate.

The PI3K-Akt kinase signalling cascade is activated in response to the activation of a wide range of receptors, including those for growth factors and G-protein coupled receptors.([570]) In response to ligand-receptor binding, the p85 regulatory subunit of PI3K is phosphorylated, which results in the activation of the p110 catalytic subunit of PI3K, which in turn phosphorylates the membrane lipid, phosphatidylinositol 3,4-biphosphate([571]) to phosphatidylinositol 3,4,5-triphosphate,([572]) which then recruits Akt (also known as PKB) to the membrane. Upstream kinases such as 3-phosphoinositide-dependent protein kinases 1 and 2 (PDK1 and 2) then phosphorylate and activate Akt at Thr-308 and Ser-473.([573])

Figure 1.5: Scheme Outlining the Mechanisms by Which the Activation of the PI3K-Akt and Raf1-MEK1/2-Erk1/2 Kinase Cascades Protect Against Lethal Reperfusion Injury, at the Time of Reperfusion. Agents which protect at the time of reperfusion bind to receptors, which via the small G-protein, RAS, activate the PI3K-Akt and Raf1-MEK1/2-Erk1/2 kinase cascades, which together comprise the Reperfusion Injury Salvage Kinase (RISK)-pathway. Protection against lethal reperfusion injury is then mediated by the: (1) phosphorylation and inactivation of pro-apoptotic factors, caspases 3 and 9, BIM, BAX, BAD and p53, one consequence of which is to prevent the release of mitochondrial cytochrome C in response to an apoptotic stimulus (shown by dashed arrows); (2) phosphorylation and activation of eNOS (endothelial nitric oxide synthase), producing nitric oxide which may protect by inhibiting opening of the mitochondrial permeability transition pore (mPTP); (3) phosphorylation and activation of p70S6K and p90S6K which can protect by inactivating BAD or regulating protein transcription/translation; and (4) regulating the expression of genes concerned with cellular survival.



The activation of Akt phosphorylates a diverse array of substrates, influencing numerous cellular processes, many of which are anti-apoptotic in action. Downstream targets of PI3K-Akt which mediate anti-apoptotic effects include (see figure 1.5):

1. Pro-apoptotic factors: Akt can phosphorylate and inactivate several pro-apoptotic factors including BAD,([574]) Bax,([575;576]) BIM,([577]) p53,([578]) and pro-caspase 9;([579])

2. Mitochondrial cytochrome C release: It has been demonstrated that signalling through the PI3K-Akt kinase cascade can inhibit apoptosis by preventing mitochondrial cytochrome C release;([580])

3. p70S6K: Akt can phosphorylate and activate this kinase which can in turn phosphorylate and inactivate the pro-apoptotic factor, BAD([581])

4. Mitochondrial Raf-1: Akt via PKC can activate mitochondrial Raf-1 which has been shown to phosphorylate and inactivate BAD.([582])

5. Gene transcription factors: Akt phosphorylates and activates IKK-α, which leads to the activation and translocation of NF-κB to the nucleus, where it acts as a transcription factor for a variety of survival pathways.([583]) Akt can phosphorylate and inhibit Forkhead transcription factor-1 mediated transcription of death-inducing genes such as Fas ligands.([584])

Other downstream targets of PI3K-Akt include (see figure 1.5):

1. Endothelial nitric oxide synthetase (eNOS): Akt phosphorylates and activates eNOS, which has been implicated in cellular protection.([585])

2. Glycogen synthase kinase-3β: phosphorylation and inactivation of glycogen synthase kinase-3β has been implicated in preconditioning-induced protection.([86])

3. GLUT4 vesicles: Matsui and colleagues([586]) have demonstrated that the constitutive over-expression of Akt enhanced sarcolemmal expression of Glut-4 and increased glucose uptake.

4. Protein kinase C (PKC): Akt can phosphorylate and activate PKC, which has been implicated in preconditioning-induced protection.([587])

1.4.3.3 The Raf-MEK1/2-Erk1/2 Kinase Signalling Cascade

The Raf-MEK1/2-Erk1/2 kinase cascade is a member of the mitogen-activated protein kinases (MAPKs), a family of serine-threonine kinases concerned with regulation of cell proliferation, differentiation and survival. Raf-1 is a serine threonine kinase which is activated in response to a variety of extra-cellular stimuli which target receptor tyrosine kinases and G-protein coupled

receptors.([192]) Activated Raf-1 then phosphorylates and activates the MEK1/2-Erk1/2 kinase cascade.([588])

The activation of Erk1/2, like Akt, can phosphorylate a diverse array of substrates, influencing numerous cellular processes, some of which are anti-apoptotic in action. Downstream targets of MEK1/2-Erk1/2 which mediate anti-apoptotic effects include (see figure 1.5):

1. Pro-apoptotic factors: Erk1/2 can phosphorylate and inactivate several pro-apoptotic factors including BAD,([574]) Bax,([577]) BIM,([577]) and caspase 3,([589])

2. Inhibiting cytochrome C-induced apoptosis: It has been demonstrated that Erk 1/2 rendered cells resistant to cytochrome C-induced apoptosis.([590])

3. p70S6K: Erk1/2 can phosphorylate and activate this kinase which in turn can phosphorylate and inactivate the pro-apoptotic factor, BAD,([591])

4. p90RSK: Erk1/2 can phosphorylate and activate this kinase which in turn can phosphorylate and inactivate the pro-apoptotic factor, BAD.([592]) Furthermore, p90RSK has been linked to the regulation of the gene expression of: (1) cAMP-response element-binding protein, which transcribes genes concerned with cellular survival;([593]) (2) regulation of protein synthesis by phosphorylation of polyribosomal proteins and glycogen synthase kinase-3; and (3) stimulation of the Na^+-H^+ exchanger.([594])

From the available evidence therefore, it appears that BAD, BAX, p70S6K and eNOS appear to be the downstream components responsible for mediating the protection associated with the activation of these kinase cascades at the time of reperfusion.

1.4.3.4 Agents which precondition versus those which protect at reperfusion: Do the PI3K-Akt and Erk1/2 cascades constitute a common pathway of protection?

It is interesting to note that these kinase cascades have also been implicated in mediating the protection associated with the phenomenon of ischaemic preconditioning.([81;82;194]) In this scenario, the activation of the kinase cascades occurs prior to the ischaemic insult and acts as a preconditioning trigger and/or mediator for cardio-protection.([595])

In the light of this evidence, one can propose that these kinase cascades may constitute a common pathway of cardio-protection, mediating the protection associated with both IPC and the RISK-pathway. Evidence in support of this proposition, is provided by the fact that agents

which precondition, such as bradykinin and AMP579,([596;597]) also confer protection when given at reperfusion.([494;524]). Conversely, agents which have been demonstrated to protect at reperfusion by activating the RISK-pathway, such as insulin, urocortin and CT-1 have also been shown to precondition the myocardium.([83;391;598])

The evidence would tend to suggest that the pro-survival kinase cascades may therefore constitute a common pathway, mediating the cardio-protection induced by IPC on the one hand, as well as protecting the myocardium through their recruitment at the time of reperfusion, on the other hand. However, the only direct evidence for this rests with insulin-induced cardio-protection, and therefore more research is needed to elucidate whether the pro-survival kinase cascades actually constitute the common pathway for cardio-protection in these two settings.

1.4.3.5 Cross-Talk Between the PI3K-Akt and Raf1-MEK1/2-Erk 1/2 kinase cascades

The previous section demonstrates that the activation of the kinase cascades which comprise the RISK-pathway, at the time of reperfusion, can protect the heart against lethal reperfusion injury. The exact interplay that exists between the PI3K-Akt and Raf-MEK1/2-Erk1/2 kinase cascades in mediating their protective effect at reperfusion is unclear, and their interaction at this time has not been previously examined. In various immortal cell lines, these kinase cascades have been demonstrated to exhibit a variety of complex, sometimes contradictory, interactions which enable mutual amplification or inhibition of the signal.

For example: (i) The small G-protein Ras can stimulate both the PI3K-Akt and Raf-MEK1/2-Erk1/2 kinase cascades;([599]) (ii) It has been demonstrated that the PI3k-Akt cascade can both inhibit([600;601]) and facilitate([602-604]) the Raf-MEK1/2-Erk1/2 kinase cascade; (iii) The Raf-MEK1/2-Erk1/2 pathway can activate the PI3K-Akt-p70S6K cascade;([605;606]) and (iv) Signalling through these kinases cascades may converge on a distal target, such as the pro-apoptotic protein, BAD.([607;608])

The phenomenon of 'cross-talk' observed between the kinase cascades, in which the inhibition of one kinase cascade results in the activation of the other and vice versa, has been observed in other organ tissue, including the lens([609]) and neuronal cells.([603]) In previous studies, using immortal cell lines, it has been demonstrated that Akt inhibits the Raf-MEK1/2-Erk1/2 kinase cascade by phosphorylation and inactivation of Raf at Ser^{259}.([600;601]) and this inhibitory pathway may be recruited at different stages of cell development,([600]) or vary

according to the concentration and type of ligand exposure.([604]) Therefore, the inhibition of the PI3K-Akt pathway may activate the Raf-MEK1/2-Erk1/2 cascade, providing a form of 'compensatory regulation'.

1.4.4 'Ischaemic Post-Conditioning'

At the Hatter Institute 3rd International Workshop on Cardio-protection, in August 2001,([610]) Jakob Vinten-Johansen first introduced the concept of 'ischaemic post-conditioning',([611]) a phenomenon which describes the myocardial protection against lethal reperfusion injury, obtained from applying brief intermittent episodes of ischaemia/reperfusion immediately *after* the index ischaemic period.([611]) Using the in vivo canine model of ischaemia-reperfusion injury, Vinten-Johansen and colleagues([611]) demonstrated that immediately following a 3 hour period of left anterior descend artery occlusion, the application of three 30 second episodes of alternate left anterior descend artery re-occlusion and reperfusion, produced a significant reduction in infarct size from 25±3% to 14±2%, an effect comparable to ischaemic preconditioning. Furthermore, neutrophil accumulation, lipid peroxidation (an indicator of oxidant stress injury), and endothelial dysfunction were all attenuated in the 'ischaemic post-conditioned' group.([611])

Interestingly, the concept of 'ischaemic post-conditioning' may not be an entirely novel phenomenon, as it may simply constitute a variation of controlled reperfusion, which several previous studies have demonstrated can protect the myocardium against lethal reperfusion injury.([612-614]) In 1986, Okamato and colleagues,([612]) demonstrated using the canine in vivo model of ischaemia-reperfusion, that graded low-pressure reperfusion could protect against lethal reperfusion injury as shown by a reduction in infarct size and improved contractile functional recovery. Peng and colleagues,([613]) using an in vivo pig model of ischaemia-reperfusion found that controlled reperfusion preserved certain parameters of mitochondrial function such as mitochondrial oxidative phosphorylation. The Vinten-Johansen group([614]) demonstrated in the canine experimental model that following the index ischaemic event, gradual reperfusion could protect the myocardium against lethal reperfusion injury as evidenced by a reduction in infarct size and attenuation of endothelial dysfunction.

Since its first description, the phenomenon of 'ischaemic post-conditioning' has been reproduced by other laboratories and in other animal experimental models and the mechanism of protection is currently being investigated with preliminary data having been presented at the

last AHA 2003 Scientific Sessions. Downey's laboratory([615]) reproduced 'ischaemic post-conditioning' in the in vivo rabbit model of ischaemia-reperfusion injury, and found the protection to be sensitive to PD 98059 (the MEK1/2 inhibitor) and L-NAME (the antagonist of nitric oxide synthase), which may suggest a role for both the MEK1/2-Erk1/2 kinase cascade and nitric oxide in mediating the protection.

Vinten-Johansen's group([616]) were able to reproduce 'ischaemic post-conditioning' in the in vivo rat model, in which they demonstrated that the 'ischaemic post-conditioning' had to be applied at the immediate onset of reperfusion, as delaying to after 1 minute reperfusion was ineffective. The protection observed in the in vivo rat model was smaller than in the other animal models with 'ischaemic post-conditioning' reducing infarct size from 52% to 40%, and the protection was shown to be associated with an attenuation in the production of ROS at reperfusion.([616])

Interestingly, the same group demonstrated the phenomenon of 'ischaemic post-conditioning' in neonatal rat myocytes by applying an 'hypoxic post-conditioning' protocol following a 3 hr episode of lethal hypoxia.([617]) This resulted in less necrotic cell death, attenuated ROS production and reduced mitochondrial calcium loading.([617])

The term 'ischaemic post-conditioning' is probably a misnomer, as the word conditioning implies that the process prepares the myocardium for the index ischaemic event, as in ischaemic preconditioning. The term modification of lethal reperfusion injury, may be more appropriate as it may transpire that 'ischaemic post-conditioning' protects the myocardium against lethal reperfusion injury, by recruiting the same mechanisms outlined in the previous sections such as attenuating ROS production and reducing mitochondrial calcium load.([617])

1.4.5 Protecting the Human Myocardium Against Ischaemia-Reperfusion Injury

Several clinical studies have examined the potential benefits of the use of various cardio-protective pharmacological agents as adjuncts to current reperfusion strategies such as thrombolysis and cardiac surgery.

1.4.5.1 Insulin

In 1962, Sodi-Pallares and colleagues first introduced the concept that glucose-insulin-potassium (GIK) may offer cardio-protection in response to ischaemia-reperfusion injury.([618])

Several inconclusive studies followed in the 1960's and 1970's, and the use of GIK as a cardio-protective strategy fell out of favour. However, attention was again directed to the benefits of GIK therapy in ischaemia-reperfusion injury, following the publication in the late 1990's of: a meta-analysis reporting a 21% significant reduction in mortality,([619]) and the DIGAMI clinical trial in which glucose and insulin therapy demonstrated a 29% significant reduction in mortality;([620]) as well as the ECLA clinical trial which reported a 66% reduction in mortality in diabetic patients reperfused following an acute myocardial infarction.([621]) Recent data has also suggested that GIK therapy in the setting of coronary artery bypass graft (CABG) surgery, may also be cardio-protective.([622])

However, in the light of both experimental ([623]) and clinical ([624]) studies that have reported findings to the contrary, and in the absence of large-scale clinical trial data, the routine use of GIK therapy in the setting of an acute myocardial infarction is currently only recommended in diabetics.([625]) A large randomised clinical trial (called GIK II) which aims to recruit up to 10,000 patients is now underway examining the potential benefits of GIK therapy following an acute myocardial infarction.([626]) Whether the mechanism of insulin-induced protection is due to the activation of the pro-survival kinases as outlined in section 1.4.3.1.a is unclear at present.

1.4.5.2 Magnesium

Several studies have reported that iv magnesium can protect the animal heart against ischaemia-reperfusion injury.([627-629]) However, clinical studies have produce mixed results with the Second Leicester Intravenous Magnesium Intervention Trial (LIMIT-2) trial([630]) reporting a benefit but the larger fourth International Study of Infarct Survival (ISIS-IV) trial([631]) reporting no benefit with intravenous magnesium given as an adjunctive therapy to thrombolysis. The disparity between the two studies may relate to the timing of the intravenous magnesium infusion which was instituted before thrombolysis in the LIMIT-2 trial but was given after thrombolysis in ISIS-IV.

1.4.5.3 Adenosine

The Acute Myocardial Infarction STudy of ADenosine (AMISTAD) demonstrated that the administration of adenosine as an adjunct to thrombolysis resulted in a 67% relative reduction in infarct size in patients with anterior myocardial infarction, as determined by technetium sestamibi single-photon emission computed tomography (SPECT) imaging.([632])

1.4.5.4 Na^+-H^+ Exchanger Inhibitors

Initial studies with the use of Na^+-H^+ exchanger as an adjunct to primary PTCA in patients with an acute myocardial infarction were positive reporting attenuated CKMB release, improved ejection fraction and improved regional left ventricular wall motion.([633]) A subsequent larger study called the ESCAMI trial (Evaluation of the Safety and Cardio-protective Effects of Eniporide in Acute Myocardial Infarction), however reported no attenuation in CKMB release in patients given the Na^+-H^+ exchanger inhibitor, eniporide as an adjunct to reperfusion therapy for an acute myocardial infarction.([634]) In retrospect, the negative outcome of these studies may have been anticipated on the basis that most of the experimental studies demonstrating a protective effect with this class of drug, had administered the Na^+-H^+ exchanger inhibitor either before or after the onset of ischaemia.([477])

In this regard the GUARDIAN (Guard during ischaemia against necrosis) trial([635]) reported that in three groups of patients at risk of a myocardial infarction (unstable angina, PTCA, and cardiac surgery) the treatment with the Na^+-H^+ exchanger inhibitor, cariporide, produced a reduction in the combined end-point of death and MI, in the sub-group of patients awaiting cardiac surgery only. The absence of a beneficial effect in the other two patient groups may be due to the fact that the reperfusion therapy in these groups was not instituted in a timely manner.([477])

1.4.5.5 Activating the Mitochondrial K_{ATP} Channel at the Time of Reperfusion

Pharmacological opening of the mitochondrial K_{ATP} channel at the time of reperfusion, following an episode of lethal ischaemia has been shown in the in vivo canine([268;269]) and swine([636]) models of ischaemia-reperfusion injury to reduce infarct size. Based on a retrospective study by Sugimoto and colleagues,([637]) demonstrating reduced cardiac events, with intravenous nicorandil (a mitochondrial K_{ATP} channel opener) given at the time of primary PTCA for myocardial infarction, the same investigators have embarked on a prospective study

investigating the benefits of opening the mitochondrial K_{ATP} channel at the time of reperfusion termed the J-WIND trial.

1.4.5.6 Free Radical Scavengers

Clinical studies investigating the role of oxidative stress as a mediator of lethal reperfusion injury in man have been largely disappointing. Human recombinant superoxide dismutase when used as an adjunct to reperfusion therapy in patients presenting with an acute myocardial infarction resulted in no improvement in left ventricular function or outcome.([638]) Large scale clinical trials of antioxidants have produced mixed results.([639-642])

In this section, the factors that mediate lethal reperfusion injury and the interventions that provide protection against lethal reperfusion injury have been reviewed. Many of these factors have a direct effect on the subject of the next section, the mitochondrial permeability transition pore (mPTP).

1.5 The Mitochondrial Permeability Transition Pore (mPTP)

1.5.1 The Discovery of the mPTP

The mitochondrial permeability transition pore (mPTP) denotes the term given to the proteinaceous pore of the inner mitochondrial membrane which on opening mediates the mitochondrial permeability transition (mPT), which describes the abrupt increase in permeability of the inner mitochondrial membrane, which takes place in response to various inducing factors.([7-10])

The concept of an actual inner mitochondrial membrane proteinaceous pore (mPTP) was first made in the late 1970's in the seminal studies by Haworth and Hunter,([7-10]) and was originally described as a "Ca^{2+}-Induced Membrane Transition in Mitochondria." But the description of the phenomenon that would later become known as the mPT, preceded that by almost 30 years, with the study by Potter in 1947, who identified Ca^{2+} as an inhibitory factor of P_i uptake (indicating ATP production) in kidney homogenates.([643]) However, it was not until 1955 that Hunter and Ford([644]) ascribed the loss of mitochondrial membrane impermeability to the mPT, in order to explain the various phenomena previous investigators had observed that mitochondria underwent when exposed to certain conditions, such as: (1) swelling in isotonic media; (2) Ca^{2+}-induced: uncoupling, ATPase activity, swelling, inhibition of site I respiration; (3) protection by ethylene diamine tetracetic acid (EDTA); (4) P_i-induced uncoupling and swelling; (5) Permeation by sucrose; (6) Loss of K^+; (7) Loss of adenine and pyridine nucleotides;(8) Loss of Kreb's cycle intermediates;(9) Loss of Co-enzyme A.

1.5.2 Characterisation of the Mitochondrial Permeability Transition Pore

By 1980, Haworth and Hunter had already discovered and characterised many of the key features of the mPTP.([7-10;645]) In 1976, they demonstrated that the mPTP was *unselectively permeable* to H^+, Ca^{2+} Mg^{2+}, choline, glucose, sucrose, and NAD(P)H.([7;9]) They also demonstrated that mPTP opening was a *reversible process*, in that the open mPTP could be completely re-sealed by adding ATP plus Mg^{2+} or by chelating Ca^{2+} with Ethylene Glycol-Tetra Acetate (EGTA).([7;9]) They determined that mPTP opening could be activated by the allosteric binding of Ca^{2+} at the matrix side of the pore, and could be inhibited by ADP and Mg^{2+}.([9;645]) The redox couples $NAD(P)^+$/NAD(P)H([7-10]) and pH([9]) were shown to modulate mPTP opening.

Based on the finding that the mPTP could be opened by atractyloside([8]) and closed by bongekrekic acid,([8]) both known inhibitors of the adenine nucleotide translocase (ANT), and that mPTP opening was sensitive to ADP,([7-10]) they proposed that the ANT might be part of the mPTP. They also anticipated that the mPTP may form at contact sites between the inner and outer mitochondrial membranes.([10]) Subsequent studies have demonstrated in both these cases that their postulations on the components of the mPTP to be true (see section 1.5.3).

Using dextrans of different molecular weight, they determined the molecular cut-off to be <1500 Da,([9]) consistent with the induction of a large pore of discrete size. Subsequent studies by Crompton's group examining the permeability of the mPTP to [^{14}C] solute fluxes, calculated that the mPTP had a diameter of 2.0-2.6 nm.([452;646]) There is evidence for a lower-conductance state of the mPTP,([647;648]) which was also anticipated by Hunter and Haworth.([9;10]) The transient (low-conductance) opening of the mPTP is discussed in section 1.5.6.

Since the initial discovery of the mPTP, an ever increasing number of inducers and inhibitors of mPTP opening have been described. Tables 1.2 and 1.3, list the important known inducers and inhibitors along with their mechanisms of action if known.

Table 1.2: List of Important mPTP Inducers. Please see text for details.

Important mPTP Inducers	***Mechanism of Action (if known)***
Ca^{2+}	Binding to Ca^{2+}-activating sites on the ANT induce mPTP opening
P_i	Increases Ca^{2+} loading and prevents matrix acidification.
Oxidative Stress and Thiol Agents	Cross-linking of inner membrane protein thiol groups induces mPTP opening
Pyridine Nucleotides	NAD+ and NADP induce mPTP opening
Fatty Acids	Mitochondrial uncoupling effect induces mPTP opening
ANT Ligands Atractyloside Carboxyatractyloside	These ligands inhibit ANT and favour the 'c' conformation which induces mPTP opening
Mitochondrial Uncouplers	Collapse of the mitochondrial membrane potential or ROS induce mPTP opening
Mitochondrial Membrane Depolarisation	Collapse of the mitochondrial membrane potential induces mPTP opening as the mPTP is voltage-sensitive

Table 1.3: List of Important mPTP Inhibitors. Please see text for details.

Important mPTP Inhibitors	***Mechanism of Action (if known)***
Pharmacological Agents	
Cyclosporin-A	By binding to cyclophilin D it prevents the interaction of the latter with the ANT
Sanglifehrin-A	This drug inhibits the cis-trans isomerase activity of cyclophilin D such that it does not modulate ANT conformation.
Trifluoperazine	? Modification of voltage-sensitivity of the mPTP
β-aminoketone derivatives	Inhibition of mPTP opening by targeting VDAC ?mechanism
Cations Mg^{2+}, Sr^{2+}, Mn^{2+}, Ba^{2+}	These divalent ions compete for the Ca^{2+}-binding sites thereby inhibiting mPTP opening
Protons	Acidic matrix pH (<7.0) inhibits mPTP opening by reversible protonation of histidine residues
Pyridine Nucleotides	NADH and NADPH inhibits mPTP opening
Adenine Nucleotides ADP/ATP	Binding of ATP (to the cytosolic side of the ANT) and ADP (to the matrix side of the ANT) inhibits mPTP opening
ANT Ligands Bongkrekic acid	This ligand inhibits ANT and favours the 'm' conformation which inhibits mPTP opening
Ubiquinine Analogues	By partially inhibiting electron flow through complex I of the electron transport chain they inhibit mPTP opening

1.5.2.1 Inducers of mPTP Opening

1.5.2.1.a Calcium and Phosphate

Mitochondrial matrix Ca^{2+}, which is a critical pre-requisite for mPTP opening, is believed to induce mPTP opening by binding to two activating sites on the matrix side of the pore.([9;649]) Ca^{2+}-induced mPTP opening can be facilitated in the presence of mPTP inducers such as P_i, a weak acid which allows the accumulation of mitochondrial Ca^{2+}up-take,([650]) and prevents matrix acidification.([651])

1.5.2.1.b Oxidative Stress

Opening of the mPTP can be induced by: (1) Reactive oxygen species generated by the mitochondrial electron transport chain, xanthine oxidase, and NADPH oxidase; (2) Exogenous sources of ROS;([652]) and (3) Oxidising agents such as t-butylhydroperoxide.([653])

Oxidative stress induces mPTP opening by:

(1) oxidising critical thiol groups of the inner mitochondrial membrane (IMM) to form thiol crosslinkage.([654;655]) Bernardi's group([654;655]) have identified one thiol group which is sensitive to the oxidation of glutathione and another thiol group which is regulated by the redox state of NAD(P). Because of the role of thiol groups in regulation of mPTP opening, thiol agents such as phenylarsenine oxide,([656]) hydroperoxides([653]) and diamide([657]) induce mPTP opening by cross-linking inner membrane thiol groups. One candidate protein of the IMM which may have to be cross-linked for mPTP opening is the adenine nucleotide translocase (ANT).

(2) oxidising $NAD(P)^{+}H$ and NAD(P)H, antagonises the inhibitory effect of these pyridine nucleotides on mPTP opening.([9])

Also, oxidative stress can induce leakiness of the IMM, independent of the mPTP, by causing lipid peroxidation of the membrane.

1.5.2.1.c Thiol Agents

Thiol agents such as diamide and phenylarsenine oxide induce mPTP opening by cross-linking critical thiol groups present on the ANT.([655]) Halestraps' group([658]) have postulated that these agents induce mPTP opening by cross-linking Cys160 and Cys257 of the ANT.

1.5.2.1.d Pyridine Nucleotides

Oxidation of the mitochondrial NAD(P)H pool induces mPTP opening.([8;659]) NADH is believed to act synergistically with ADP to inhibit mPTP opening,([645]) and therefore its oxidation removes this inhibitory effect on mPTP opening.

1.5.2.1.d Fatty Acids

Fatty acids diffuse into the mitochondria as the protonated electroneutral form, thereby effectively uncoupling mitochondria,([660]) the effect of which would be to induce mPTP opening, as the latter is voltage-sensitive.([661])

1.5.2.1.e Adenine Nucleotide Translocator Ligands

ANT ligands such as atractyloside and carboxyatractyloside,([9]) which stabilise the ANT in the 'c' conformation (with the adenine nucleotide binding site facing the cytosol) favour mPTP opening.([662]) Conversely, ANT ligands such as bongkrekate and acyl-CoA's, which stabilise the ANT in the 'm' conformation (with the adenine nucleotide binding site facing the matrix) inhibit mPTP opening.([9;662])

1.5.2.1.e Mitochondrial Uncouplers and Mitochondrial Membrane Depolarisation

Haworth and Hunter were the first to demonstrate that mitochondrial uncoupling could induce mPTP opening,([8]) providing the first clue that mPTP opening may be regulated by voltage. Mitochondrial uncouplers induce mPTP opening by collapsing the mitochondrial membrane potential. The mPTP is believed to be voltage-sensitive and Bernardi's group([651;661]) have proposed that the modulation of the mPTP by matrix Ca^{2+}, ADP, protons, Mg^{2+}, fatty acids, and cross-linking of thiol groups may all be mediated by the shifting of the voltage sensitivity of the mPTP to opening. They demonstrated that the rate of mPTP opening in response to different doses of the uncoupler, FCCP, could be modified by agents known to modulate mPTP opening.([651;661])

1.5.2.2 Inhibitors

1.5.2.2.a Cyclosporin-A

In 1987, Fournier and colleagues,([663]) while examining the effect of the immunosuppressive agent, cyclosporin-A (CsA) on mitochondrial function discovered that this agent could inhibit mitochondrial Ca^{2+} efflux and allowed mitochondria to accumulate large amounts of Ca^{2+}. Crompton's group([664]) and Pfeiffer's group([665]) both realised the significance of these findings and proceeded to demonstrate that CsA was a potent inhibitor of mPTP opening a year later. This discovery would have a major impact on the research into this area, especially in terms of elucidating the molecular composition of the mPTP (see section 1.5.3.).

Using a chromatography affinity binding column, Halestrap's group([666]) demonstrated that a fusion protein between cyclophilin D and glutathione-*S*-transferase (GST) bound to ANT in response to the thiol, diamide, and binding was inhibited by CsA, suggesting that CsA inhibited mPTP opening by preventing the binding of cyclophilin D to ANT.([666])

CsA can inhibit mPTP opening caused by a variety of mPTP inducers but its inhibition is not absolute.([667;668]) Furthermore, CsA-induced inhibition of mPTP opening can be overcome by high mitochondrial [Ca^{2+}],([649;669-672]) supporting the proposition by Lemaster's group of the existence of an unregulated CsA-insensitive form of mPTP opening (see section 1.5.3).([673])

In addition, as well as inhibiting mPTP opening it can inhibit the protein phosphatase, calcineurin through its binding to cyclophilin A, which has resulted in the use of CsA analogues such as N-methyl 4-valine CsA([674]) and N-methyl-4-iosleucine-CsA (NIM811),([675]) which inhibit mPTP opening without inhibiting calcineurin. In this regard, a new mPTP inhibitor called sanglifehrin-A has been described which also inhibits mPTP opening without inhibiting calcineurin.([676])

1.5.2.2.b Sanglifehrin-A

Halestrap's group([676]) have recently characterised a new mPTP inhibitor called sanglifehrin-A (SfA), which is a newly described immunosuppressant which differs from CsA in that it does not inhibit calcineurin.([677;678]) Halestrap's group([676]) demonstrated that the mechanism of mPTP inhibition also differed from that of CsA. SfA was demonstrated to bind to and inhibit the PPIase activity of cyclophilin D (K_i of about 2.2 nmol/l) but unlike CsA,([666]) SfA did not prevent the binding of cyclophilin D with ANT.([676]) Using the isolated perfused rat heart they demonstrated that inhibiting mPTP opening using SfA (at 1.0 µmol/l) protected the heart against ischaemia-reperfusion injury, as evidenced by attenuated LDH release and improved recovery of left ventricular contractile function.([676])

1.5.2.2.c Trifluoperazine

The phenothiazine, trifluoperazine was originally thought to inhibit mPTP opening by competing with Ca^{2+} for its binding site on the mPTP.([679]) However, it also has many non-specific effects which include inhibition of calmodulin and it also inhibits the F_1F_0ATPase.([680]) Later studies have suggested that it may modify the voltage sensitivity of the mPTP.([681])

1.5.2.2.d A New Class of VDAC Inhibitors: the β-aminoketone derivatives

Bernardi's group([682]) have recently described a new class of mPTP inhibitors, called *β-aminoketone derivatives,* which appear to target the VDAC. The most potent mPTP inhibitor of

this class of compounds was Ro 68-3400, with a potency comparable to that of CsA.([682]) The mechanism by which they inhibit mPTP opening is currently unknown and is under investigation.

1.5.2.2.e Divalent Cations

Divalent cations such as Mg^{2+}, Sr^{2+}, Mn^{2+}, Ba^{2+} can inhibit mPTP opening by competing with Ca^{2+} for its activating site on the mPTP.([679;683]) Alternatively, these ions may inhibit mPTP opening by binding to an alternative site on the cytosolic side of the mPTP.([679])

1.5.2.2.f Protons

The protective effect of protons against mPTP opening was first recognised in 1953, when it was demonstrated that mitochondrial swelling did not take place at pH<6.0.([684]) Haworth and Hunter demonstrated that protons inhibited mPTP opening by competing for the Ca^{2+} activating site on the mPTP,([9]) a finding which was later confirmed.([685]) A later study by Bernardi's group suggested that the proton-mediated inhibition of mPTP opening was due to the protonation of histidyl residues on the matrix side of the mPTP.([686])

1.5.2.2.g Adenine Nucleotides

The binding of ATP (to the cytosolic side of the ANT) and the binding of ADP (to the matrix side of the ANT) inhibits mPTP opening. Binding of ADP competes for the Ca^{2+}-activating site on the mPTP.([9])

1.5.2.2.h Ubiquinone Analogues

Bernardi's group([687-689]) have demonstrated that substrates which increase electron flow through the complex I of the electron transport chain compared to complexes II and IV, sensitise the mPTP to Ca^{2+}, and therefore ubiquinone analogues which act to inhibit electron flow through complex I can inhibit mPTP opening.

1.5.2.2.i Pyruvate and Propofol

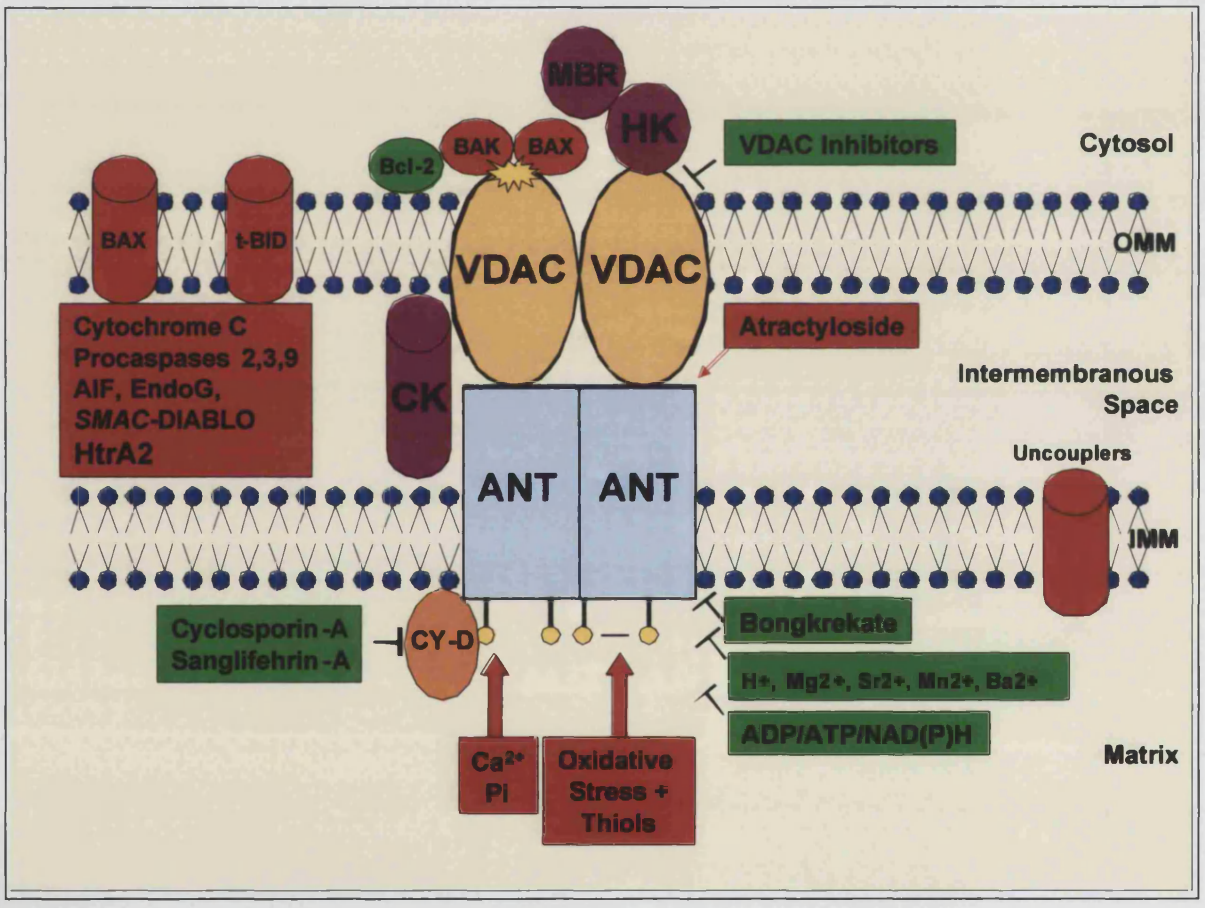
Halestrap's group have demonstrated that both pyruvate (possibly by lowering intracellular pH and scavenging free radicals)([690]) and propofol (a free radical scavenger)([691]) protected the isolated perfused rat heart against ischaemia-reperfusion injury by inhibiting mPTP opening, as assessed by the mitochondrial 2-deoxyglucose entrapment technique.

1.5.3 The Identification of the mPTP

The concept of a proteinaceous pore of the inner mitochondrial membrane mediating the mitochondrial permeability transition (mPT), was first postulated in studies by Haworth and Hunter, in which they demonstrated the all-or-nothing nature of the mPT, and in which they suggested the opening of a pore in the inner membrane mediated the mPT.([8;9]) The discovery by Crompton's group that mPTP opening could be immediately interrupted by EGTA([452;692-694]) and that it could be inhibited by cyclosporin-A([664]) added support to this hypothesis. Until that point, the mPT was believed to be due to membrane permeability defects upon accumulation of lysophospholipids.([695]) In 1989, definitive evidence for the existence of a pore was provided from electrophysiological patch-clamp studies which identified a large 1.3 nS (in 150 mmol/l KCl) multi-state channel in the mitochondrial inner membrane-the mitochondrial megachannel (MMC),([696;697]) which was later identified as the mPTP.

The actual molecular composition of the mPTP is currently unknown but the most popular working model suggests that it comprises three core components: the adenine nucleotide translocase (ANT) of the inner mitochondrial membrane (IMM), the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane (VDAC) and cyclophilin D of the mitochondrial matrix. The mPTP is believed to form when these three components associate, which has been demonstrated to occur at contact points between the OMM and IMM. Figure 1.6 is a cartoon demonstrating the basic structure of the mPTP, and its interaction with the various inducing and inhibitory agents as well as the pro- and anti-apoptotic members of the Bcl-2 family (see later section). Hunter and Haworth had postulated that the mPTP may form at fusion points between the OMM and IMM.([645])

Figure 1.6: Schematic Representation of the Mitochondrial Permeability Transition Pore. The mPTP, which comprises the core components of the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane (OMM), the adenine nucleotide translocase (ANT) of the inner mitochondrial membrane (IMM), cyclophilin D (CY-D) of the mitochondrial matrix, is under the regulatory control of a multitude of factors. The mPTP can be regulated by additional components such as the creatine kinase (CK) of the intermembranous space and the mitochondrial benzodiazepine receptor (MBR) and hexokinase (HK). mPTP opening can be induced by the pro-apoptotic members of the Bcl-2 family, BAX and BAK. In addition, BAX and truncated BID (t-BID) may form pores in the OMM and cause the release of pro-apoptotic factors such as cytochrome C, pro-caspases 2, 3 and 9, apoptosis-inducing factor (AIF), Smac/DIABLO, high temperature requirement A2 (HtrA2), and endonuclease G (Endo G), independent of mPTP opening. The anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-X_L are believed to inhibit mPTP opening and stabilise the OMM. Inducers of mPTP opening include Ca^{2+}, P_i, oxidative stress and thiol agents (which cross-link thiol groups on ANT), uncouplers, and atractyloside (which inhibits ANT). Inhibitors of mPTP opening include cyclosporine-A and sanglifehrin-A (which inhibit cyclophilin D activity), bongkrekate (which inhibits ANT), divalent cations, Mg^{2+}, Sr^{2+}, Mn^{2+}, Ba^{2+}, protons (which protonate histidyl residues on the ANT), ADP/ATP and NADH, and VDAC inhibitors.



1.5.3.1 Components of the mPTP

1.5.3.1.a Adenine Nucleotide Translocase

When Haworth and Hunter described the mPTP for the first time in 1979, they proposed that the ANT might be part of the mPTP, based on their findings that mPTP opening was sensitive to ADP, and the ANT inhibitors atractyloside and bongkrekic acid.([7-10;645]) In 1988, Le Quoc and

Le Quoc([662;698]) confirmed the involvement of the ANT in mPTP formation and proposed that mPTP inducers such as atractyloside, carboxyatractyloside, a high NADP/NADPH ratio, and fatty acids stabilised the 'c' conformation (where the nucleotide-binding site is on the cytosolic side) of the ANT and mPTP inhibitors such as bongkrekic acid and matrix ADP stabilised the 'm' conformation (where the nucleotide-binding site is on the matrix side).([662;698]) Modulation of thiol groups on the ANT were demonstrated to mediate transitions between the two conformations.([698])

The studies appeared to suggest that the 'c' conformation of ANT could deform into a non-selective pore under high [Ca^{2+}].([698]) In 1990, Halestrap's group([699]) first proposed and have subsequently demonstrated that cyclophilin D induces the conformational change in ANT to form the mPTP (see section 1.5.3.1.b).

In 1996, Brustovetsky & Klingenberg,([700]) demonstrated that reconstituting purified ANT into liposomes created a selective anti-porter, which converted to a non-selective pore under high [Ca^{2+}], that possessed features in common with the mPTP. For example, it was demonstrated to be sensitive to pH (with half-maximal activity at pH 6.2), voltage (sensitivity over the range 150-180mV), bongkrekic acid and ADP. Interestingly, however, it was found to be insensitive to carboxyatractyloside.([700])

1.5.3.1.b Cyclophilin D

The finding that CsA could inhibit mPTP opening in 1988,([664;665]) suggested the involvement of cyclophilin in the formation of the mPTP. In 1990, Halestrap's group([699]) were the first to propose that cyclophilin D may mediate the conformational change in the ANT to form the mPTP. Cyclophilin D is a cis-trans peptidyl prolyl transisomerase (PPIase) which can mediate conformational changes in proteins. Evidence confirming the role of cyclophilin D in mPTP formation include:

(1) Mitochondria have been demonstrated to contain a CsA-sensitive PPIase,([699]) which has been purified and cloned.([701-703])

(2) The relative potencies of cyclosporin-A, G and H as inhibitors of PPIase correlate with their ability to inhibit mPTP opening.([699;704;705])

(3) Similar amounts of bound CsA (approximately 50 pmol/mg of mitochondrial protein) are needed to block the mPTP and to inhibit the PPIase activity of cyclophilin D.([699;706])

(4) Cyclophilin D has been demonstrated to bind to the IMM in response to thiols and oxidative stress, factors known to induce mPTP opening.([670;671]) Interestingly, an increased mitochondrial volume was demonstrated to increase cyclophilin D binding to the inner mitochondrial membrane in response to Ca^{2+}, though the significance of this finding is unclear.([671])

In contrast, there is evidence to suggest that cyclophilin D is not required for mPTP opening at very high mitochondrial [Ca^{2+}], such that mPTP opening is no longer inhibited by CsA.([649;669-672;704;707])

1.5.3.1.c Interaction Between the ANT and Cyclophilin D

Using a chromatography affinity binding column, Halestrap's group([666]) demonstrated that a fusion protein between cyclophilin D and glutathione-*S*-transferase (GST) bound to ANT in response to the thiol, diamide, and binding was inhibited by CsA. However, a similar study by Crompton's group demonstrated binding of cyclophilin D with both VDAC and ANT, and that the binding was not inhibited by CsA,([708]), wherein lies the reason for disparate views between the two groups concerning the actual composition of the mPTP, with Crompton's group supporting a role for VDAC in the formation of the mPTP, and Halestrap's group holding the contrary view.

In view of the findings by Bernardi's group([655]) that mPTP opening is regulated by cross-linking of critical thiol groups on the inner mitochondrial membrane, Halestrap's group([658]) have been investigating the cross-linking of three thiol groups (Cys^{57}, Cys^{160} and Cys^{257}) present on the matrix side of ANT, which may regulate mPTP opening. They demonstrated that PAO and diamide induce mPTP opening by cross-linking the thiols, Cys^{160} and Cys^{257}, present on the matrix side of ANT, causing inhibition of ADP binding to the mPTP.([658])

Halestrap's group([699]) postulated that cyclophilin D binds to the ANT via Pro^{62}, a proline residue present on the matrix side of ANT, adjacent to Cys^{57}. In apparent support of this proposition, this proline residue is not present in yeast mitochondrial ANT and mPTP opening in this setting is not sensitive to CsA.([672])

1.5.3.1.d Voltage-Dependent Anion Channel

The VDAC, first characterised in 1979,([709]) is a membrane protein that forms an aqueous channel in the OMM. At voltages <30 mV, it displays a high conductance formation (4 nS at 1M KCl) with a diameter of 4 nm, and is anion selective. At voltages >30 mV, it displays a lower conductance of 2 nS with a diameter of 2 nm and the ion selectivity changes to cations. ([709;710])

The mPTP has been proposed to form at contact sites between the IMM and OMM.([645]) Studies have demonstrated the increased presence of VDAC and ANT at these contact sites.([711-713]) Crompton's group demonstrated that the fusion protein between cyclophilin D and GST bound a 1:1 VDAC-ANT complex.([708]) This cyclophilin D-VDAC-ANT complex was incorporated into lysosomes and demonstrated mPTP activity in response to Ca^{2+} and P_i which was blocked by CsA.([708])

1.5.3.1.e Other Components of the mPTP

Several other components have been postulated to form part of the mPTP, but the evidence suggests that these additional components probably contribute to the other mitochondrial roles the VDAC-ANT complexes at the contacts sites are involved in. For example, hexokinase([714]) is bound to the junctional complexes to facilitate the entry of mitochondrial generated ATP into the first step of glycolysis.([715]) Creatine phosphokinase (present in the intermembranous space) associates with the junctional complexes to facilitate energy transfer between ADP (which is poorly diffusible in the cytosol) and the more diffusible creatine-creatine-phosphate couple in the cytosol.([714]) Furthermore, creatine kinase can form octamers which by binding to the VDAC-ANT channel can suppress mPTP opening.([716;717]) This finding may be of importance in the setting of ischaemia-reperfusion injury, in which Garlid has proposed that preserving the integrity of the mitochondrial intermembranous space, during myocardial ischaemia and maintaining the octameric structure of CK, is critical to the protection induced by opening the mitochondrial K_{ATP} channel.([159]) It has been postulated that the VDAC and ANT can interact in such a way that these cytosolic kinases can communicate with ANT and regulate oxidative phosphorylation.([718]) The benzodiazepine receptor may also associate with the VDAC to facilitate the transfer of cholesterol across the mitochondrial membranes.([719]) Members of the Bcl-2 family such as Bcl-2 and BAX have also been demonstrated to be associated with components of the mPTP and are discussed in section 1.5.4.2.a.

Recently, Lemaster's group([673]) have proposed a new model for the mPTP which dispenses with the concept of a pore and postulates that the mPTP is formed by aggregation and misfolded integral membrane proteins damaged by oxidants and other stresses, which are blocked by chaperone proteins. In this scenario, the mPTP displays both a regulated Ca^{2+}-dependent CsA-sensitive opening as well as an unregulated Ca^{2+}-independent CsA-insensitive opening.([673]) In support, of this model which does not implicate a role for the ANT, mPTP

conductance has been reported in triple ANT knockout yeast,([720]) and high conductance pores can be induced in the absence of ANT by other mitochondrial carrier proteins such as the aspartate/glutamate and phosphate carriers.([721;722])

1.5.4 Consequences of mPTP Opening

The conditions required to induce mPTP opening are usually encountered when the cell is subjected to stress such as during ischaemia-reperfusion injury. In this setting, mPTP opening occurs, and results in both apoptotic and necrotic cell death. In this scenario, the mPTP opening that occurs is a high-conductance non-selective opening, in contrast to the transient low-conductance mPTP opening which can occur under physiological conditions. The latter is discussed in section 1.5.6, whereas this section will focus on the high-conductance non-selective opening which results in cell death.

1.5.4.1 Necrotic Cell Death and the mPTP

Under aerobic conditions, the oxidation of pyruvate in the tricarboxylic acid cycle in the mitochondrial matrix generates reduced coenzymes (NADH and FADH), which transfer their electrons into the electron transport chain. The electron flow through the electron transport chain releases energy, which is used to translocate protons from the mitochondrial matrix via the electron transport carriers (I, III and IV) and into the intermembranous space. This creates an electrochemical gradient into the mitochondria which drives protons back into the matrix. The only entry route into the mitochondria is via the F_0F_1-ATPase complex, which utilises the energy to phosphorylate ADP, producing ATP. Therefore, maintaining the relative impermeability of the inner mitochondrial membrane is critical to ATP production, as it permits the generation of this electro-chemical gradient which drives ATP phosphorylation-the chemiosmotic hypothesis first described by Mitchell & Moyle, in the 1960's.([723])

Therefore, the abrupt permeation of the IMM that occurs on opening of the mPTP, creates a bypass circuit for the entry of protons into the mitochondrial matrix, which dissipates the proton-mediated mitochondrial membrane potential. This halts ATP production and results in ATP hydrolysis as the F_0F_1-ATPase complex breaks down ATP in an attempt to maintain the mitochondrial membrane potential. Therefore, if mPTP opening is prolonged and is not reversed, ATP depletion results followed by necrotic cell death. Opening of the mPTP is associated with

loss of matrix Ca^{2+}, depletion of reduced glutathione, depletion of NADPH, and the hypergeneration of superoxide anion. Prolonged opening of the mPTP in the setting of ischaemia-reperfusion results in necrotic cell death and is dealt with in section 1.5.5.

1.5.4.2 Apoptotic Cell Death and Mitochondria

Another major consequence of mPTP opening is apoptotic cell death. Apoptosis is an evolutionary conserved ATP-dependent process that permits the selective removal of cells through an intrinsic cell-suicide program, and is characterised by DNA fragmentation, cell shrinkage without loss of membrane integrity or inflammation.([724]) Apoptosis is orchestrated by caspases (cysteinyl-aspartate-specific-proteinases) that are constitutively inactive and become activated after proteolytic cleavage. Until 1996, caspase activation was believed to occur only via a receptor-mediated pathway.([725]) However, it is now clear that there exists a mitochondria-dependent pathway of caspase activation.

A pivotal study by Liu and colleagues in 1996,([726]) first demonstrated that mitochondria played a role in apoptosis. They showed that caspase activation by apoptosis protease-inducing factor-1 (APAF-1) in a cell-free system required both dATP and mitochondrial cytochrome C.([726]) Since this publication, several other pro-apoptotic proteins have been demonstrated to be present in the mitochondrial intermembranous space including pro-caspases 2, 3 and 9,([727;728]) apoptosis-inducing factor (AIF, which mediates DNA fragmentation),([729]) second mitochondrial activator of caspases (Smac, which inhibits cytosolic inhibitors of apoptosis [IAP's])([730]) also known as direct IAP-binding protein with low pI (DIABLO),([731]) high temperature requirement A2 (HtrA2, a serine protease that binds to and inhibits IAP's),([732]) and endonuclease G (mediates DNA fragmentation).([733])

The cytochrome C released into the cytosol binds to APAF-1 and procaspase 9 to form an apoptosome, which then cleaves caspase 9 into its active form, thereby setting off the caspase cascade and apoptosis.([734;735])

However, the mechanism by which mitochondrial cytochrome C and the other pro-apoptotic factors translocate from the mitochondrial intermembranous space into the cytosol is currently under debate, since the process would require large holes or breaks in the OMM-so called mitochondrial membrane permeabilisation (MMP). Two mechanisms have been postulated: (1) Pro-apoptotic and anti-apoptotic members of the Bcl-2 family interact with

components of the mPTP to mediate MMP; and (2) Pro-apoptotic members of the Bcl-2 family mediate MMP, independent of the mPTP.

The Bcl-2 family contains both: (1) pro-apoptotic members comprising (a) the Bax subfamily, Bax, Bak and Bok and (b) the BH3-only proteins, Bid, Bad and Bim; and (2) anti-apoptotic family members Bcl-2 and Bcl-x_L.([736]) The anti-apoptotic proteins Bcl-2 and Bcl-x_L reside at the mitochondria and stabilise the OMM, thereby preventing MMP. The pro-apoptotic proteins Bax, Bid, Bim and Bad reside in the cytosol and in response to an apoptotic stimulus they translocate to the mitochondria where they interact with the OMM to induce MMP.([736;737]) Bid is activated by caspase 8 into truncated Bid (t-Bid)([738]) which then induces the oligomerisation and activation of both Bax([739;740]) and Bak,([741]) and all three are then able to insert into the OMM to induce MMP and apoptosis. At the mitochondria, Bim([742]) and Bad([743]) induce apoptosis by interacting with both Bcl-2/Bcl-x_L to antagonise their anti-apoptotic activity and Bax/Bak.([744]) Bid is believed to induce MMP independently of the mPTP([745]) but whether Bax and Bak act via the mPTP or not is still a matter of debate.([746;747])

1.5.4.2.a The mPTP is Required to Mediate Apoptotic Cell Death

In response to an apoptotic stimulus the opening of the mPTP renders the IMM non-selectively permeable to molecules up to 1500 Da. Matrix proteins larger than this size remain in the mitochondria and exert a colloidal osmotic pressure, which results in the influx in water and mitochondrial matrix swelling. The IMM, because it is folded into cristae has a larger surface area when compared to the OMM, so the IMM can accommodate the increase in matrix volume, whereas the OMM ruptures, releasing the pro-apoptotic factors that reside in the mitochondrial intermembranous space-also called mitochondrial membrane permeabilisation (MMP)([748]) Therefore, in this model pro-apoptotic factors such as Bax and Bak are believed to induce MMP via interacting with components of the mPTP.

Evidence in support of this model includes:

(1) Inhibiting MMP using mPTP inhibitors such as cyclosporin-A or bongkrekic acid have been demonstrated to inhibit apoptotic cell death,([749-752]) suggesting that mPTP opening is an obligatory mediator of apoptosis.

(2) Ultrastructural evidence for matrix swelling and rupture of the OMM has been obtained in many models of cell death.([750]) However, mitochondrial swelling and OMM rupture is not a

universal feature of apoptosis suggesting alternative mechanisms of OMM (see next section).([740;741;753;754])

(3) In isolated mitochondria, Bax or Bak can induce MMP which is sensitive to mPTP inhibitors such as CsA and bongkrekate.([755-757]) Interestingly, at low doses Bax was demonstrated to induce permeabilisation of the OMM only, though at higher doses it also induced the permeabilisation of the IMM.([755])

(4) In cells, apoptotic death induced by BNIP3 could be inhibited by CsA and bongkrekate.([758])

(5) Narita and colleagues([757]) have demonstrated that Bax and Bak can bind to VDAC, and induce mPTP opening which is sensitive to CsA and bongkrekic acid. Shimizu and colleagues([759]) showed that Bax and Bak could induce opening of the VDAC, thereby allowing the exit of mitochondrial cytochrome C, whereas the anti-apoptotic protein, Bcl-x_L acts to close the VDAC and suppress apoptosis. Furthermore, VDAC-1 deficient cells were resistant to Bax/Bak induced apoptosis.([759]) The pore formed by the interaction between Bax and VDAC had a conductance four and ten times greater than those of VDAC or Bax channel, respectively.([760]) Crompton's group have also demonstrated co-immunoprecipitation of Bax with VDAC and ANT.([761])

(6) In other studies Bax has been demonstrated to co-immunoprecipitate with ANT,([762]) and Bax and ANT reconstituted into synthetic lipid bilayers have been found to generate channels.([762]) Furthermore, the channel formed by the interaction between Bax and ANT was larger than that formed by the mPTP alone.([763])

(7) Several viral and bacterial proteins which induce apoptosis interact with components of the mPTP such as: (a) Vpr from HIV-1 and vMIA/UL37 from cytomegalovirus both of which bind to ANT;([764;765]) and (b) hepatitis virus BX-protein and porin B from Neisseria meningitidis both of which bind to VDAC.([766;767])

(8) An interesting recent study by Ichas' group demonstrated that 'flickering' of the mPTP and cytochrome C release, signals for Bax translocation to the mitochondria.([768])

The problem with this model is that mPTP opening in this setting will result in ATP depletion which would be expected to halt the energy-dependent apoptotic process, so it appears counter-intuitive to have mPTP opening in apoptotic cell death. Furthermore, mitochondrial swelling and OMM rupture does not always occur in apoptotic cells, and mitochondrial cytochrome C release can occur independent of mPTP opening.([741;753;754;769])

Therefore, MMP may occur by a direct action of pro-apoptotic protein on the OMM, independent of the mPTP.

1.5.4.2.b The mPTP is Not Required to Mediate Apoptotic Cell Death

Recombinant Bax and Bid can form ion channels in artificial membranes.([770]) It has been demonstrated that Bax forms pores in liposome that are large enough to release cytochrome C.([771]) Recombinant t-BID added to isolated mitochondria, has been reported to cause MMP without inducing mitochondrial membrane depolarisation and the MMP was not affected by mPTP inhibitors.([772]) In support of this cytochrome C can be released from mitochondria that have a preserved ultrastructure and conserved mitochondrial membrane potential.([740;754;769])

Therefore, under pathological conditions the opening of the mPTP may result in both apoptotic and necrotic cell death. One such pathological condition is in the setting of ischaemia-reperfusion where the mPTP has been demonstrated to be a critical determinant of cell death.

1.5.5 The Role of the mPTP in Ischaemia-Reperfusion Injury

Crompton's group were the first to postulate a role for the mPTP in the setting of ischaemia-reperfusion injury.([694]) Evidence supporting the role of the mPTP in ischaemia-reperfusion comprises three lines of investigation: (1) The major known inducers of mPTP opening, mitochondrial [Ca^{2+}] and [P_i], oxidative stress, and ATP depletion are all present in the setting of ischaemia-reperfusion; (2) Studies have demonstrated mPTP occurring in the setting of ischaemia-reperfusion injury; and (3) Inhibiting mPTP opening in the setting of ischaemia-reperfusion using pharmacological mPTP inhibitors such as CsA can protect the heart against ischaemia-reperfusion injury.

1.5.5.1 Metabolic and Biochemical Changes During Ischaemia-Reperfusion Predispose to mPTP Opening

The adenine nucleotides, ATP and ADP are known to inhibit mPTP opening and therefore in the setting of ischaemia,([9;645]) when there is a depletion of adenine nucleotides, the reduction in ATP and ADP level removes the inhibitory effect of these adenine nucleotides on mPTP

opening. ATP is the more potent mPTP inhibitor than ADP,([773]) and cells become vulnerable to mPTP opening when more than two-thirds of cellular ATP has been lost.

During ischaemia, the rise in cytosolic Ca^{2+} begins when about two-thirds of cellular ATP has been depleted. A study([774]) has demonstrated that providing the cellular Ca^{2+} does not exceed 1-2 µmol/l during the anoxic/ischaemic period, mitochondrial function can recover on reoxygenation/reperfusion. However, if this threshold is exceeded then cell death and presumed mPTP opening occurs. This limit is close to the set-point of 1-3 µmol/l at which cytosolic Ca^{2+} results in mitochondrial Ca^{2+} loading and mPTP opening,([775]) suggesting that the mitochondrial $[Ca^{2+}]$ is a critical determinant of mPTP opening and cell death.([466]) Concomitant with ATP depletion, mitochondrial Ca^{2+} loading results in mPTP opening.

1.5.5.2 Timing of mPTP Opening in Ischaemia-Reperfusion Injury

Studies have demonstrated that despite the prevailing factors for mPTP induction being present during the index ischaemic period, it is during the first few minutes of reperfusion that mPTP opening is most likely to occur.([52;470;776;777]) It is believed that during the index ischaemic period, mPTP opening is inhibited by the lactate-induced acidic pH, and high levels of ADP and Mg^{2+}. Several events then take place at reperfusion that induce mPTP opening in the first few minutes of reperfusion: (1) with washout of the lactate and activation of the Na^{+}-H^{+} exchanger, the pH returns to physiological levels and mPTP opening is no longer inhibited by the acidic conditions; (2) there is a burst of oxidative stress from within the mitochondria due to reoxygenation of a reduced electron transport chain; (3) repolarisation of the mitochondrial membrane drives the influx of Ca^{2+} into the mitochondria.

Halestrap's group([776]) demonstrated using the isolated perfused rat heart that mPTP opening (measured by the mitochondrial entrapment of 2-deoxyglucose-see section 1.5.7.3) occurs in the first few minutes of reperfusion. Di Lisa's group([777]) demonstrated the same finding using the CsA-sensitive loss of mitochondrial NAD+, that occurred during the first few minutes of reperfusion to indicate mPTP opening. However, it must be borne in mind, that damage to cellular and mitochondrial membranes induced during necrotic cell death at the time of reperfusion may result in the release of mitochondrial NAD+, independent of mPTP opening.

At the cellular level, Le Masters' group([470]) demonstrated mPTP opening occurring at the time of reoxygenation, in hepatocytes subjected to period of simulated ischaemia. In this model mPTP opening was indicated by the entry of the membrane-impermeant fluorescent dye

calcein (see section 1.5.7.2.a) and mitochondrial membrane depolarisation.([470]) Marban's group([52]) demonstrated a CsA-sensitive increase in mitochondrial Ca^{2+} (indicating mPTP opening), occurring at the time of reoxygenation, in myocytes exposed to a sustained period of simulated ischaemia.

In contrast to these findings a recent study by Brown's group suggested that mPTP opening occurred during the index ischaemic period.([778]) However, in this study mPTP opening was not assessed directly, and was only implied by the CsA-sensitive mitochondrial cytochrome C release that occurred during ischaemia.

1.5.5.3 Inhibiting mPTP Opening During Ischaemia-Reperfusion Using CsA

Opening of the mPTP during ischaemia-reperfusion is a critical determinant of cell death, a fact which is supported by the studies demonstrating that pharmacologically inhibiting its opening is protective.

Following the discovery that CsA acted as a potent inhibitor of mPTP opening in 1988,([664]) studies have demonstrated that inhibiting mPTP opening in the setting of ischaemia-reperfusion using CsA can protect against cell death. In 1991, Crompton's group([779]) demonstrated using rat myocytes subjected to anoxia-reoxygenation injury, that cyclosporin-A at a concentration of 0.2 µmol/l protected the cell against necrotic cell death (assessed by Trypan-blue exclusion and LDH release) and protected against the loss of ATP. Importantly, they discovered that the protection by CsA was dependent on the concentration, with the best protective effect demonstrated at concentrations between 0.2-0.4 µmol/l, and a concentration >1.0 µmol/l conferring no protection.([779]) This dose-dependent response to CsA has been observed in subsequent studies.([780]) The mechanism for the variation in response to CsA according to the concentration is unknown, but may relate to the non-specific effects of CsA such as the inhibition of other cellular cyclophillins. Halestrap's group([780]) demonstrated using the isolated perfused rat heart that CsA could protect the heart against ischaemia-reperfusion injury (as measured by LDH release and recovery of left ventricular contractile function) and could preserve the ATP/ADP ratio even if CsA was given at the time of reperfusion. Several subsequent studies have demonstrated that inhibiting mPTP opening using CsA is cardio-protective.([236;773;781-784])

Studies have demonstrated that CsA can protect hepatocytes subjected to high Ca^{2+} and oxidative stress.([785-787]) This was confirmed in studies by Le Master's group, ([470]) who

demonstrated that CsA administered at the time of reoxygenation protection hepatocytes against anoxic-reoxygenation injury by inhibiting mPTP opening. Subsequently, Ashraf's group([251]) demonstrated that CsA could protect neonatal rat myocytes subjected to anoxia-reoxygenation injury against both apoptotic and necrotic cell death.

In 1992, Shiga and colleagues([788]) first demonstrated that CsA could protect the brain against ischaemia-reperfusion injury. This finding has been confirmed in several subsequent studies in protection against ischaemia-reperfusion injury,([789-793]) and protection against hypoglycaemic damage.([794;795]) Yoshimoto and colleagues([796]) reported that the protection induced by CsA was more marked if it was given at the time of reperfusion of ischaemic brain, and protection was also associated with preservation of Akt phosphorylation.

CsA has also been recently demonstrated to protect human atrial muscle harvested at the time of cardiac surgery, subjected to hypoxia-reoxygenation,([797]) and has been demonstrated to protect skeletal muscle against ischaemia-reperfusion injury.([798])

Recent studies have suggested that CsA may protect against ischaemia-reperfusion injury by mechanisms other than via inhibition of mPTP opening. For example, Chen and colleagues([799]) demonstrated that CsA protected against oxidative stress-induced apoptosis by producing ROS, and up-regulating HSP70 and iNOS, factors that have been associated with preconditioning-induced protection. Niemann and colleagues([800]) have demonstrated that the reduction in infarct size induced by pre-treating rats with CsA for 3 days prior to ischaemia-reperfusion correlated with a dose-dependent reduction in myocardial energy metabolism. A recent study attributes the protection of CsA against ischaemia-reperfusion injury to its ability to attenuate the cell volume increase induced by ischaemia.([801]) Furthermore, Downey's group([802]) demonstrated that CsA could protect the isolated perfused rabbit heart against ischaemia-reperfusion injury by inhibiting calcineurin.

Therefore, because of the non-specific effects of CsA, it is necessary to evaluate the effect of other mPTP inhibitors in ischaemia-reperfusion injury. In this regard, CsA analogues such as N-methylvaline 4-CsA, which does not inhibit calcineurin but does inhibit mPTP opening have been demonstrated to protect the cell against both apoptotic and necrotic cell death.([674;705;776])

1.5.5.4 Inhibiting mPTP Opening During Ischaemia-Reperfusion Using Other Agents

Halestrap's group([676]) have characterised and investigated the effect of sanglifehrin-A (SfA) on mPTP opening in the setting of ischaemia-reperfusion injury. This mPTP inhibitor does not inhibit calcineurin and was demonstrated to protect the isolated perfused rat heart against ischaemia-reperfusion injury (as measured by LDH release and recovery of left ventricular contractile function).([676]) Furthermore, SfA given at the time of reperfusion was demonstrated to protect the isolated perfused rat heart subjected to ischaemia-reperfusion injury by inhibiting mPTP opening, detected by the mitochondrial 2-deoxyglucose technique.([803])

The phenothiazine, trifluoperazine, a known mPTP inhibitor has been demonstrated to protect the heart([804-808]) and brain([809;810]) against ischaemia-reperfusion injury but these studies did not demonstrate directly that the protection was due to inhibition of mPTP opening. Le Master's group([653]) did demonstrate that oxidative stress-induced mPTP and cell death could be prevented by trifluoperazine.

Halestrap's group have also demonstrated that inhibition of mPTP opening (detected by the mitochondrial entrapment of 2-deoxyglucose) using either pyruvate([690]) or probofol([811]) protected the isolated perfused rat heart against ischaemia-reperfusion injury.

1.5.5.4 Inhibiting mPTP Opening During Ischaemia-Reperfusion: the pH Paradox

Opening of the mPTP during the ischaemic period is inhibited by the prevailing acidic conditions. On reperfusion, with the restoration of physiological pH at the time of reperfusion, cell death ensues probably secondary to mPTP opening. Studies have demonstrated that reperfusing with acidic buffer can protect the heart against lethal reperfusion injury-the so-called pH paradox.([469;470])

1.5.6 Transient (Low-Conductance) mPTP Opening

Studies from the mid-1980's appeared to suggest that the mPTP may exist in two different open configurations: (1) The high-conductance (<1500 Da) non-selective state of mPTP opening as discussed in the previous section;([9]) and (2) a low-conductance (<300 Da) ion-selective (Ca^{2+}, H^+ and K^+) state of mPTP opening (also called transient mPTP opening or 'mPTP flicker'), which is the less well known of the two, and which may occur under physiological circumstances.([647;648;812])

Naturally, in 1979, the pioneers of the mPTP, Haworth and Hunter had already anticipated the existence of mPTP 'flicker': "the Ca^{2+}-induced permeability is discontinuous in time: the treated mitochondria must be continuously switching between a permeable and an impermeable state".([9]) Furthermore, they had observed a sub-conductance state of the mPTP, when they found that mitochondria impermeable to sucrose could still release Ca^{2+} at a fast rate, in a ruthenium-red insensitive manner, and in the absence of Na^+.([10])

1.5.6.1 Characteristics of Transient (Low-Conductance) mPTP Opening

Early studies suggested that in the presence of Ca^{2+} and P_i, mitochondria exhibited an initial H^+-specific mitochondrial permeability increase, followed by a non-selective mitochondrial permeability increase.([649;693;813]) Interestingly, numerous studies had described a Na^+ and ruthenium red-insensitive pro-oxidant-induced Ca^{2+}-efflux, which may represent the low-conductance form of mPTP opening.([812;814])

Ichas & Mazat confirmed that the transitory low-conductance mPTP opening was permeable to Ca^{2+}, H^+ and K^+ but was impermeable to sucrose (suggesting a molecular cut-off of 300 Da), and did not cause mitochondrial swelling.([647;815]) They demonstrated that mPTP opening resulted in a transitory depolarisation of mitochondrial potential and mitochondrial Ca^{2+} efflux, followed by mitochondrial repolarisation and reaccumulation of Ca^{2+}, indicating that mPTP opening was transitory.([647]) The low-conductance state of mPTP opening has been demonstrated to precede the high-conductance mPTP opening in several studies.([648;816])

In electrophysiological studies, it has been demonstrated that the mPTP can display several sub-conductance levels, including a half-conductance state of 500 pS mPTP flicker,([817;818]) which may correspond to the transient low-conductance mPTP open configuration.

Balakirev & Zimmer([819]) described in rat liver mitochondria, that the mPTP opening induced by phenylarsenine oxide occurred gradually, with an electrogenic K^+ influx initially, followed by a permeability to protons. Interestingly, the K^+ influx was demonstrated to not be sensitive to glibenclamide.

Brustovetsky & Dubinsky([820]) demonstrated in isolated brain mitochondria that a Ca^{2+} challenge induced two forms of mPTP opening depending on the substrate. When energized with succinate plus glutamate, a Ca^{2+} challenge induced mitochondrial membrane depolarisation and an increase in mitochondrial Ca^{2+} and an increase in matrix volume, though when energized with succinate alone, the mitochondrial accumulated Ca^{2+} without mitochondrial

swelling. Interestingly, the latter form of mPTP opening was sensitive to CsA but neither form of mPTP opening was sensitive to carboxyatractyloside nor bongkrekic acid, suggesting that the ANT was not involved. ([820])

However, some studies have described low conductance channels in the inner membrane which do not appear to be due to mPTP opening. Broekemeier and colleagues([821]) have described a small pore of the inner mitochondrial membrane which is proton-selective and is insensitive to CsA, and so whether it forms part of the mPTP is unclear at present. Furthermore, Kushnareva & Sokolove([822]) have described a pro-oxidant Ca^{2+}-sensitive induced low-conductance inner membrane channel which was permeable to Ca^{2+} and not H^{+}, and was insensitive to the normal mPTP inhibitors, CsA, trifluoperazine, and Mg^{2+}.

1.5.6.2 Regulation of Transient (Low-Conductance) mPTP Opening

Transient (low-conductance) mPTP opening is inhibited by CsA, acidic pH, and is activated by matrix Ca^{2+}, alkaline pH, mitochondrial membrane depolarisation and oxidative stress.([647;659;815]) Ichas & Mazat have postulated a mechanism for transitory mPTP opening in the context of mitochondrial calcium induced calcium release (mCICR), that appears to be *pH-operated*: A Ca^{2+} signal reaches the mitochondria and Ca^{2+} entry occurs driven by the electrochemical potential, which increases matrix pH (mediated by charge compensation via the respiratory chain), which triggers mPTP opening, resulting in mitochondrial membrane depolarisation. This results in mitochondrial calcium efflux via the mPTP and matrix acidification, which closes the mPTP, allowing restoration of the mitochondrial membrane repolarisation and Ca^{2+} entry, and so the cycle continues resulting in mPTP flickering.([647])

Vercesi's group([659;823;824]) demonstrated that the *oxidation of NADH*, using acetoacetate induced a CsA-sensitive mitochondrial Ca^{2+}efflux in the absence of any change in respiration or mitochondrial membrane potential suggesting induction of the low-conductance form of mPTP opening.

1.5.6.3 Detecting Transient (Low-Conductance) mPTP Opening

1.5.6.3.a Mitochondria

In the original description of the mPTP, Hunter & Haworth observed a spontaneous release of Ca^{2+} from mitochondria that was inhibited by Mg^{2+}, Sr^{2+}, ADP and BSA, suggesting opening of the mPTP under steady state conditions,([10]) which may well have been due to transient (low-

conductance) mPTP opening. Crompton's group([692;693]) demonstrated that under steady state conditions sucrose entered mitochondria, suggesting mPTP opening occurring under basal conditions and under the control of matrix [Ca^{2+}]. Huser & Blatter([825]) demonstrated mPTP 'flicker' in mitochondria fixed to coverslips and loaded with the fluorescent dye, tetramethyl-rhodamine methyl ester (TMRM). The TMRM accumulates in the mitochondria according to the mitochondrial membrane potential, and on confocal laser-illumination, mitochondria undergo CsA-sensitive mPTP 'flicker' represented by cycles of depolarisation and repolarisation, as the mPTP opens and closes, respectively.([825])

Electrophysiological studies of the mPTP, in which patch-clamp measurements are made on mitochondrial mitoplasts, have demonstrated mPTP 'flicker' with various conductances across the mPTP.([826])

1.5.6.3.b Cells

Using the same TMRM model, Huser and Blatter([827]) demonstrated that the mitochondria in myocytes loaded with TMRM undergo mPTP 'flicker'. This phenomenon has also been observed in Duchen's laboratory in myocytes([828;829]) and astrocytes,([830]) and by Fall & Bennet in neuroblastoma cells.([831]) The finding that mPTP opening occurs under physiological conditions was observed in a study by Di Lisa's group([832]) in which they demonstrated in hepatocytes, the mitochondria of which had been loaded with the fluorescent dye, calcein, that there was a spontaneous reduction in mitochondrial calcein fluorescence, indicating transient mPTP opening, which was sensitive to CsA. This finding has been confirmed in myocytes.([266])

1.5.6.3.c Whole Heart

In the mitochondrial 2-DOG entrapment technique, devised by Halestrap's group([690;776;803;811;833]) for detecting mPTP opening in the isolated perfused heart (in which mPTP opening is indicated by the mitochondrial entrapment of 2-DOG), the entry of 2-DOG into mitochondria was noted under basal conditions in several studies. This may have suggested that transient mPTP opening was occurring under physiological conditions, although this was not supported by the fact that it was demonstrated to be insensitive to cyclosporin-A.([690;776;803;811;833])

1.5.6.4 Proposed Physiological Roles of the mPTP

Several physiological roles have been suggested for transient (low-conductance) mPTP opening:

1.5.6.3.a As a Calcium Release Channel

Gunter & Pfeiffer([834]) proposed that mPTP opening under physiological conditions could act as an energetically favourable mechanism for ridding mitochondria of excess solutes including Ca^{2+}, and may also provide a pathway for uptake of essential components from the cytosolic compartment without a requirement for specific transport processes. In support of its role in mediating Ca^{2+} efflux, Altschuld and colleagues([835]) demonstrated that cyclosporin-A inhibited mitochondrial efflux in adult rat myocytes under normal conditions, suggesting that Ca^{2+}-efflux via mPTP opening occurs under physiological conditions. In this scenario, a rise in mitochondrial [Ca^{2+}], which triggers mPTP opening, caused mitochondrial membrane depolarisation (allowing Ca^{2+} efflux via the mPTP), and the mPTP then closes once mitochondrial [Ca^{2+}] had returned below threshold levels, allowing repolarisation to occur.([674])

1.5.6.3.b Role in Calcium Signalling

Evtodienko([836]) observed in Ehrlich ascites tumour cells, that Ca^{2+} induced mitochondrial oscillatory changes in respiration, mitochondrial membrane potential, pH and Ca^{2+} efflux consistent with transient flickering of the mPTP. Subsequently, in a series of publications Ichas & Mazat([520;647;648;815;837-839]) established a role for mitochondrial Ca^{2+}-induced Ca^{2+} release (mCICR) dependent on transitory opening of the mPTP operating in a low-conductance mode in conveying electrical and calcium signals. Initial studies demonstrated that Ca^{2+} loading induced a CsA-sensitive mitochondrial Ca^{2+} spike via transitory opening of the mPTP which rendered the inner mitochondrial membrane permeable to Ca^{2+}, H^+, and K^+,([815]) causing a mitochondrial depolarisation spike (mDPS).([647]) Importantly, Ichas & Mazat demonstrated that a locally induced mDPS/mCICR could propagate throughout a population of mitochondria, and in this way propagate an electrical or calcium signal from one part of the cell to another.([647]) In the context of signalling, they demonstrated that during inositol 1,4,5-triphosphate (IP_3)-induced mobilisation of Ca^{2+} from the endoplasmic reticulum, the mCICR amplified the cytosolic Ca^{2+} transients.([647]) The phenomenon of mCICR has also been demonstrated in liver mitochondria by another laboratory.([816])

1.5.6.3.c To Mediate Mitochondrial ROS Release

Zorov and colleagues([840]) have demonstrated that laser-induced generation of mitochondrial ROS, via photosensitisation of the fluorescent dye, TMRM, induces mPTP opening and subsequent ROS generation-termed ROS-induced ROS release.

1.5.6.3.d To Mediate Apoptotic Signalling Within the Cell

Transitory opening of the mPTP may release mitochondrial cytochrome C,([841]) and Ca^{2+}-dependent 'waves' of mitochondrial depolarization may contribute to propagation of the apoptotic signal within the cell.([842]) Pacher & Hajnoczky([842]) demonstrated that an apoptotic stimulus initiates a Ca^{2+}-dependent 'wave' of mitochondrial depolarisation which was propagated through opening of the mPTP and was associated with cytochrome C release. A recent study by De Giorgi and colleagues([768]) has demonstrated that mPTP flicker may induce mitochondrial cytochrome C release, which signals the mitochondrial translocation of the pro-apoptotic protein Bax.

1.5.6.3.e A Role in Learning and Synaptic Plasticity

A recent study suggests that a possible physiological function of the mPTP in the brain is to contribute to learning and synaptic plasticity through mitochondrial Ca^{2+} regulation. They observed that either VDAC-deficient mice or mice treated with CsA (at a concentration directed to target cyclophilin D) displayed difficulties in learning.([843])

1.5.7 Methods for Detecting mPTP Opening

When studying the role of the mPTP, it is necessary to be able to both induce and detect it reproducibly and accurately. However, most models which detect mPTP opening depend on measuring or detecting a known effect of mPTP opening such as mitochondria membrane depolarisation or following the re-distribution of membrane-impermeant molecules such as the fluorescent dye calcein (in isolated mitochondria or intact cells) or radio-labelled de-oxyglucose (in the whole heart), either into or out of mitochondria, on mPTP opening. Which ever model is used it is important to demonstrate that the variable that is being used to represent mPTP opening is sensitive to CSA (a potent inhibitor of mPTP opening).

1.5.7.1 Detecting mPTP Opening in Mitochondria

In isolated mitochondria, the opening of the mPTP leads to:

1. *matrix swelling* which results in rupture of the outer mitochondrial membrane,
2. *the release of pro-apoptotic factors such as cytochrome C* from the intermembrane space,
3. *collapse of the mitochondrial membrane potential*,
4. *the release of small molecules up to 1500 Da from the matrix, including calcium and fluorescent dyes such as calcein*, through the mPTP.([9])

Therefore, detecting mPTP opening in this setting involves monitoring the consequences of mPTP opening.

1.5.7.1.a "Large Amplitude Matrix Swelling" of Isolated Mitochondria

Mitochondrial matrix swelling secondary to mPTP opening is detected as the reduction in absorbance (when a spectrophotometer is used) or the increase in 90° light scattering (when a spectrofluorimeter is used) at a wavelength of 545 nm.([844]) A variation of this technique was used by Haworth and Hunter in their original description of the mPTP.([7-10]) They first induced mPTP opening with Ca^{2+} and then exposed the mitochondria to polyethylene glycol (PEG) which shrunk the mitochondria, and mPTP opening was indicated by the reduction in the light scattering that occurs on exposure to PEG.([7-10]) The advantage of this technique is that it allowed the investigator to modify the mitochondrial matrix, such that the effect of mPTP inducers and inhibitors could be examined directly.

1.5.7.1.b Monitoring the Influx/Efflux of Proteins or Ions

Crompton's group measured the influx of [^{14}C]-sucrose to quantify mPTP opening in isolated mitochondria.([693]) The loss of soluble proteins such as cytochrome C and apoptotic-inducing factor (AIF) which normally reside in the intermembranous space can be measured on induction of mPTP opening.([755]) On mPTP opening, Ca^{2+} efflux can be measured using Ca^{2+}-sensitive dye or electrode.([647])

1.5.7.1.c Detecting the Collapse in Mitochondrial Membrane Potential

Opening of the mPTP dissipates the mitochondrial membrane potential which can be detected using fluorescent dyes or a $TPMP^{+}$ electrode. Using flow cytometry or confocal fluorescence microscopy,([825]) the collapse in mitochondrial membrane potential that occurs on mPTP

opening is represented by a loss in mitochondrial fluorescence of a lipophilic cationic dye. These dyes accumulate in the mitochondrial matrix, according to the electrochemical gradient, adhering to the Nernst equation,([845]) according to which every 61.5 mV increase in membrane potential corresponds to a 10-fold increase in monovalent cation concentration in the matrix. Given that the mitochondrial membrane potential is usually –120 to –170mV, the concentration of cations is 2 to 3 logs higher in the matrix than in the cytosol.([844])

1.5.7.1.d Monitoring Calcein Efflux

The fluorophore, calcein, is membrane impermeable, although mitochondria can be loaded with its membrane-permeable non-fluorescent form, calcein acetoxymethyl ester (calcein-AM). Once within the mitochondria, non-specific esterases remove the acetoxymethyl ester group generating free calcein, which is fluorescent and becomes trapped within the matrix, as it is membrane impermeable. Given the size of calcein (620 Da) it can only leave the matrix if the mPTP opens. Therefore mPTP opening is represented by the loss of mitochondrial calcein fluorescence, which can be detected either by using flow cytometry or confocal microscopy.([755;825;846])

1.5.7.1.e Electrophysiological Studies

Patch clamp studies of excised mitochondrial membranes allows the investigator to alter the composition of the medium on either side of the membrane and permits measuring of the conductance characteristics of the mPTP.([707]) Using this technique the Mitochondrial Mega Channel (MMC),([697]) which has been subsequently identified as the mPTP,([847]) was first discovered.

1.5.7.2 Detecting mPTP Opening in the Intact Cell

Until the development of these techniques, detecting mPTP opening in situ depended on monitoring secondary events such as mitochondrial membrane potential or on pharmacological tools such as CsA. However, the use of fluorescent dyes allows the direct assessment of mPTP opening, though this technique is not without its problems.([848]) Most of these models depend on being able to follow the movement of fluorescent dyes in or out of mitochondria on opening of the mPTP, and employ the use of confocal fluorescence microscopy.

1.5.7.2.a Dual Loading with TMRM and Calcein

This technique has been used extensively by Le Master's group([470;653;849;850;851]) to detect mPTP opening in both hepatocytes and myocytes. Cells are loaded with both calcein-AM which does not appear to enter mitochondria and TMRM which localises to the mitochondrial according the mitochondrial membrane potential. Opening of the mPTP is represented by (a) movement of the fluorescent dye, calcein, into the mitochondria from the cytosol and (b) loss of TMRM fluorescence on collapse of the mitochondrial membrane potential.([470;653;849;850;851])

Controversy surrounds this technique as Di Lisa's group have been unable to reproduce this technique, arguing that the apparent filling defects present when loading with calcein are due to quenching of the calcein fluorescent signal by TMRM, and do not indicate mitochondria.([832;852]) Therefore, on mPTP opening, when TMRM exits the mitochondria because of the mitochondrial membrane depolarisation, one would artifactually observe enhanced calcein fluorescence in the mitochondria, as the calcein signal is dequenched.([852])

1.5.7.2.b Loading with Calcein and Cobalt Chloride

Di Lisa's group([832;852-854]) first described this technique for localising the calcein signal to mitochondria. If cells are loaded with calcein-AM alone, the calcein signal is not localised to the mitochondria. Therefore, by quenching the cytosolic calcein fluorescence signal using cobalt chloride, one can localise the calcein signal to the mitochondria. Opening of the mPTP is represented by the reduction in mitochondrial calcein fluorescence. This reproducible and reliable technique for detecting mPTP opening has been used by several other laboratories.([266;855;856])

The disadvantage of this technique is that cobalt chloride can interfere with Ca^{2+} uptake and slightly decreases respiration.([832]) This technique was also demonstrated to detect transient (low-conductance) mPTP opening in hepatocytes([832]) and myocytes.([266]) Di Lisa's group([832]) demonstrated that mitochondrial calcein fluorescence declined under steady state conditions in a CsA-sensitive manner, indicating the occurrence of mPTP opening under physiological conditions.

1.5.7.2.c Loading with Calcein Alone

The mitochondria of cells are selectively loaded with calcein-AM, by using a method of loading that allows the cell to 'pump' out cytosolic calcein. Opening of the mPTP is represented by the

movement of calcein out of the mitochondria.([857]) The advantage of this technique is that it allows the mitochondrial loading of calcein without the use of cobalt chloride.

1.5.7.2.d Pinocytic Loading with Calcein

Cells are pinocytically loaded into the cytosol with free calcein (which is membrane impermeable). Opening of the mPTP is represented by movement of calcein into the mitochondria.([858])

1.5.7.2.e Detecting the Collapse in Mitochondrial Membrane Potential

In this technique, cells are loaded with the fluorescent dye, tetramethylrhodamine methyl ester (TMRM) which localises to mitochondria, according to the mitochondrial membrane potential. Laser-illumination of the cells generates reactive oxygen species within the mitochondria, which provoke mPTP opening, as detected by mitochondrial membrane depolarisation. This technique has been used in a number of different laboratories and represents a reproducible technique for inducing and detecting mPTP opening in the intact cell.([647;768;827-830;839;840;859;860])

However, there have been studies which suggest that the mitochondrial depolarisations observed when using TMRM fluorescence do not indicate mPTP opening, but these studies refer to the physiological oscillations in mitochondrial respiration and membrane potential, in the absence of significant oxidative stress.([861-863])

1.5.7.3 Detecting mPTP Opening in the Whole Heart

Halestrap's group([666;690;776;783;803;811]) have developed a technique for detecting mPTP opening in the isolated perfused rat heart. Rat hearts are perfused with titriated 2-deoxyglucose before being subjected to ischaemia-reperfusion injury. The 2-DOG loads into the cytosol but can only enter the mitochondria if mPTP opening occurs. Therefore, the mitochondrial entrapment of 2-DOG is used as a measure of mPTP opening.

Di Lisa's group([777]) devised an alternative method for detecting mPTP opening in the isolated perfused rat heart. The mitochondrial loss of NAD^+ is used as a measure of mPTP opening. However, because the mitochondrial loss of NAD^+ can occur under conditions of membrane damage, it may not be specific for mPTP opening. Therefore, this technique would have to be used in conjunction with the detection of mitochondrial NAD^+ loss from isolated mitochondria.

1.6 Summary and Main Objectives of the Thesis

Mitochondrial permeability transition pore (mPTP) opening is induced under pathological conditions such as ischaemia-reperfusion injury, when conditions which predispose to its opening prevail. These include a high mitochondrial [Ca^{2+}] and [P_i], ATP depletion and oxidative stress. Opening of the mPTP disrupts normal mitochondrial function and is a critical determinant of both apoptotic and necrotic cell death. Therefore, inhibiting mPTP opening during ischaemia-reperfusion injury offers a powerful target for protecting the heart against ischaemia-reperfusion injury.

The main aim of this thesis is to demonstrate that inhibiting the mPTP opening that occurs at the time of reperfusion, following a lethal period of ischaemia, is a common mechanism for cardio-protection whether it be by agents which protect when given at the time of reperfusion or by myocardial preconditioning. In Chapter 4, we examine the effect of pharmacologically inhibiting mPTP opening during the first few minutes of post-ischaemic reperfusion, the time-period when mPTP opening has been demonstrated to occur. In Chapter 5, we examine the powerful cardio-protective phenomenon that is myocardial preconditioning, and we investigate a novel mechanism of protection in this setting. Specifically we investigate whether this form of protection is mediated by the inhibition of mPTP opening at the time of reperfusion. In Chapter 6, we examine the pro-survival kinases, Akt and Erk1/2 MAPK, as a possible mechanism linking the process of myocardial preconditioning to the inhibition of mPTP opening, at the time of reperfusion. Finally, in Chapter 7, we examine the role of the mPTP as a potential mediator of myocardial preconditioning. Specifically, we examine the transient (low-conductance) form of mPTP opening, which paradoxically, does not lead to cell death and may actually benefit cellular function.

Chapter Two: HYPOTHESES

2.1 HYPOTHESIS ONE

Inhibiting the prolonged (high-conductance) mPTP opening which takes place at reperfusion is a common target for cardio-protection: irrespective of whether protection is mediated by myocardial preconditioning or by interventions applied solely at the time of reperfusion.

This first major hypothesis was divided into 3 parts:

2.1.1 Inhibiting the prolonged (high-conductance) mPTP opening that occurs at the time of reperfusion, following a lethal ischaemic insult, protects the heart against ischaemia-reperfusion injury.

2.1.2 Myocardial preconditioning protects by inhibiting the prolonged (high-conductance) mPTP opening that occurs at time of reperfusion, following a lethal ischaemic insult.

2.1.3 Myocardial preconditioning protects by inhibiting the prolonged (high-conductance) mPTP opening that occurs at time of reperfusion, via the activation of the pro-survival kinases Akt and Erk1/2.

In section 1.5.5 of the introduction an account was given outlining the role of the prolonged (high conductance) opening of the mPTP, which occurs at the time of reperfusion, as a critical determinant of cell death in the setting of ischaemia-reperfusion injury. The first objective of the study (2.1.1) was to demonstrate that pharmacologically inhibiting its opening at the time of reperfusion protects the heart against ischaemia-reperfusion injury (see chapter 4).

The next objective (2.1.2) was to determine whether the powerful protection associated with inhibiting the prolonged (high-conductance) mPTP opening at the time of reperfusion, is a common mechanism of cardio-protection and whether it contributes to the protection induced by the phenomenon of myocardial preconditioning (see chapter 5).

The next objective (2.1.3) was to explore the potential mechanisms through which myocardial preconditioning inhibits mPTP opening at the time of reperfusion. In this regard, the role of the pro-survival kinases Akt and Erk1/2 was examined (see chapter 6).

2.2 HYPOTHESIS TWO

Transient (low-conductance) opening of the mPTP mediates both preconditioning and mitochondrial uncoupling-induced protection by acting as a channel for mitochondrial ROS release.

In section 1.5.6 of the introduction, the transient (low-conductance) form of mPTP opening was described. This form of mPTP opening does not lead to cell death and may actually contribute to the protective mechanisms associated with myocardial preconditioning, by reducing mitochondrial calcium load and facilitating mitochondrial ROS release/signalling. The role of this transient (low-conductance) form of mPTP opening in myocardial protection associated with ischaemic preconditioning and mitochondrial uncoupling was examined (see chapter 7).

Chapter Three: GENERAL METHODS

3.1 Animals

Male Sprague-Dawley rats were used in this study. All animals were obtained from Charles River UK Limited, (Margate, UK), and received humane care in accordance with The Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (The Stationery Office, London, UK). Animals were allowed to acclimatise for a minimum of 4-5 days prior to use. They were kept in cages of four and had free access to fresh water and standard pellet chow (RM1 diet) and were subjected to a 12 hour light-dark cycle, and maintained at 19-22°C, and 55±10% humidity.

3.2 In Vitro Infarct Model in Rat Heart

The isolated crystalloid-perfused rat heart model of ischaemia-reperfusion was employed for this study to simulate the human pathological condition of myocardial ischaemia-reperfusion injury for several reasons: (1) it is a highly reproducible robust preparation which permits the measurement of: contractile function, biochemical markers of necrosis (such as lactate dehydrogenase and creatine kinase), morphological markers (such as infarct size) and electrophysiological markers (such as arrhythmias); (2) it allows the study of large numbers in a relatively short period of time and provides reproducible data; (3) it allows the examination of various pharmacological agents in the setting of ischaemia-reperfusion injury, (4) it removes the confounding effects of the systemic circulation (neuro-hormonal factors) and therefore allows one to examine the direct effect of interventions on the myocardium.([864])

The disadvantages of this preparation include: (1) the heart being isolated from blood-borne factors and neuronal innervation, meaning that it is unable to simulate in vivo conditions; (2) the preparation is constantly deteriorating.([864])

3.2.1 Langendorff Crystalloid-Perfused Rat Heart Model

In this study, the Langendorff perfused heart model, as first described by Oscar Langendorff in 1895,([865]) was used. Rats were anaesthetised with sodium pentobarbital (55 mg/kg intra-peritoneally, Sagatal-Rhone Merieux), and were given heparin sodium (300 IU intra-peritoneally, Multiparin CP Pharmaceuticals Ltd.) to prevent thrombus formation in the heart and vessels.

When the rat was sufficiently anaesthetised, indicated by the loss of the pedal withdrawal reflex, a trans-abdominal incision was made and the thoracic cavity was exposed by a bilateral incision along the lower rib margin. The anterior thoracic cage was then reflected superiorly, and the heart was gently held between the fingers and excised.

Immediately after excision, the heart was immersed in cold perfusion solution (at 4°C to limit ischaemic injury and to arrest the heart) and then mounted on a constant hydrostatic pressure (at 100 mmHg) Langendorff-perfusion apparatus (represented schematically in figure 3.1). The time taken from excising the heart to mounting the heart on the Langendorff apparatus was kept under one minute, to avoid inadvertently inducing ischaemic preconditioning. The constant pressure mode of perfusion was used as it allows auto-regulatory mechanisms induced by ischaemia-reperfusion to manifest, whereas a constant flow mode of perfusion, may over-ride these mechanisms.([864])

The heart's aorta was cannulated, allowing retrograde perfusion of crystalloid down the vessel, which forces the aortic valves shut and directs the perfusate into the coronary arteries. The whole myocardium is therefore perfused, with the perfusate then draining into the right atrium. The hearts were perfused with a modified Krebs-Henseleit bicarbonate buffer containing (in mmol/l): NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.7, and glucose 12.([866]) The major modification made to the original composition described by Krebs & Henseleit was the reduction in $CaCl_2$ from 2.5 to 1.7 mmol/l, as the former concentration does not take into account the calcium bound to proteins. All solutions were filtered through a Whatman 1.0-μm microfilter to remove particulate impurities and were bubbled with 95% O_2/CO_2, at a flow which maintained the pH between 7.35-7.40, as measured using a gas analyser (ABL 700, Radiometer, Denmark).

Temperature was continuously monitored by a thermo-probe inserted into an incision made at the base of the pulmonary artery and was maintained between 37.0°C and 37.5°C (figure 3.1). Temperature was controlled by use of a thermostatically-controlled water-jacketed system in which all glass reservoirs and the heart perfusion chamber are surrounded by rapidly flowing warmed water at 37.0-37.5°C, using a water circulator (figure 3.1).

A latex, fluid-filled, iso-volumic balloon was introduced into the left ventricle via the left atrium, through a hole made by removing the left atrial appendage, to monitor contractile function of the left ventricle. The balloon was inflated with distilled water to give a left ventricular end diastolic pressure of 4-8 mmHg, which was displayed on a chart recorder (Multitrace 2,

Lectromed, UK) (see figure 3.1). Left ventricular developed systolic and diastolic pressures, heart rate and coronary flow were noted at regular intervals.

A 3/0 Mersilk suture was passed under the left main coronary artery, using a round-bodied curved surgical needle, and the ends of the suture were passed through a pipette tip to form a snare (figure 3.2 depicts a heart in stabilisation). After the 30 minutes stabilisation period, the hearts were subjected to 35 minutes regional ischaemia. This was induced by occluding the left main coronary artery with the snare, thereby clamping the snare to the epicardial surface (figure 3.3 depicts a heart in regional ischaemia). Effective ischaemia was confirmed by a 50% reduction in coronary flow rate and rate pressure product.

After 35 minutes of regional ischaemia, reperfusion was initiated by releasing the ends of the suture, and the heart was perfused for 120 minutes. An increase in coronary flow upon reperfusion was indicative of successful re-flow, but coronary flow subsequently declined in all groups during the 120 minutes reperfusion period. In reperfusion the left ventricular developed pressure recovered gradually, though never reaching stabilisation values, a feature of "run-down" in this experimental procedure ([864]).

In the early part of the study, a standard constant-pressure Langendorff apparatus was employed for isolated rat heart perfusion (figure 3.1). However, in the latter part of the study, a Langendorff-apparatus (AD Instruments Ltd, Australia) devised by Professor Shattock and colleagues was used in which an electrical feedback system monitors the perfusion pressure and maintains the delivery of a constant pressure mode of perfusion.([867]) In this set-up, haemodynamic variables were measured and displayed using a Mac Lab.

Figure 3.1: ***Schematic Representation of Langendorff-Perfusion Apparatus.*** The isolated rat heart is mounted on a steel aortic cannula and perfused in constant pressure mode (100 mmHg) with oxygenated Kreb's Henseleit buffer which is warmed as it passes through the heated water jacket. A fluid-filled latex balloon inserted into the left ventricle monitors the left ventricular pressure, which is displayed on the chart recorder. A thermo-probe inserted into the pulmonary artery monitors temperature. The coronary effluent is measured as an indicator of the coronary flow rate.

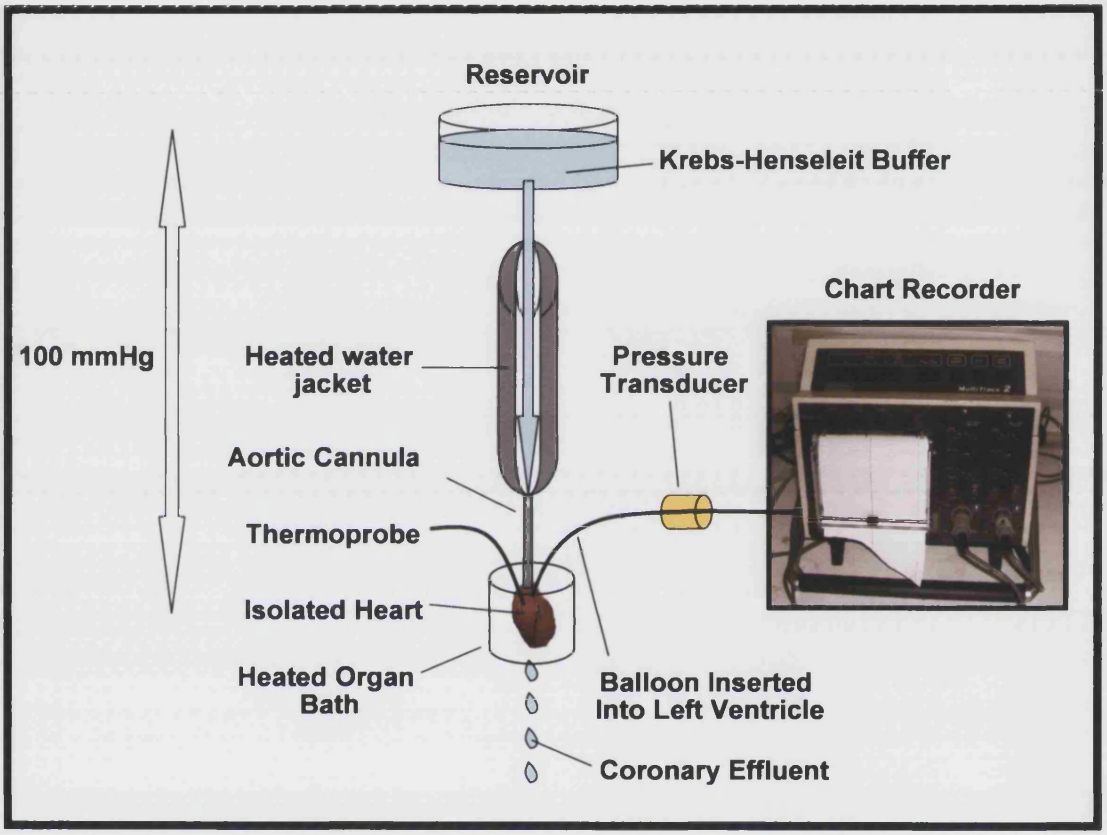


Figure 3.2: ***Isolated Perfused Rat Heart in Stabilisation:*** with loose snare and plastic pipette tip positioned around the left coronary artery.

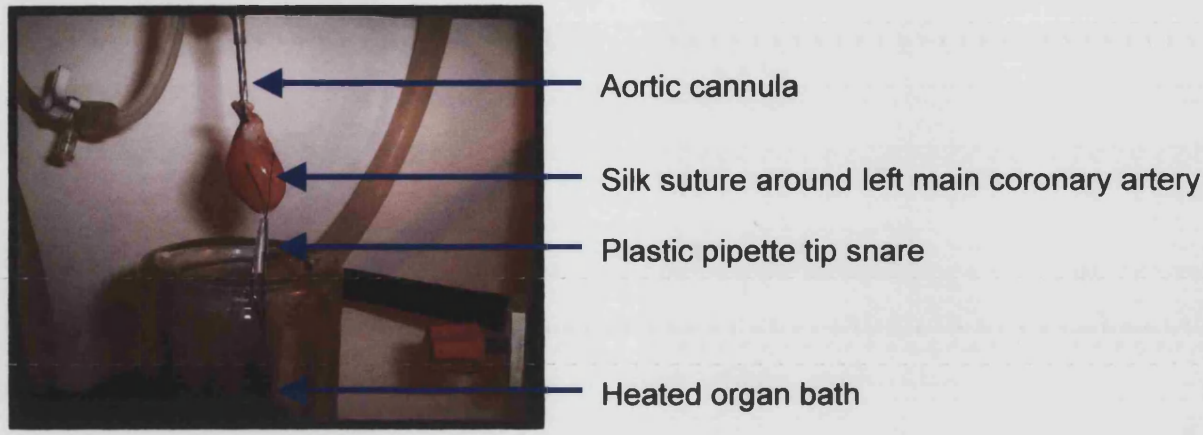


Figure 3.3: ***Isolated Perfused Rat Heart in Ischaemia*:** with suture pulled tight and held in place by plastic pipette tip snare, to induce regional ischaemia.

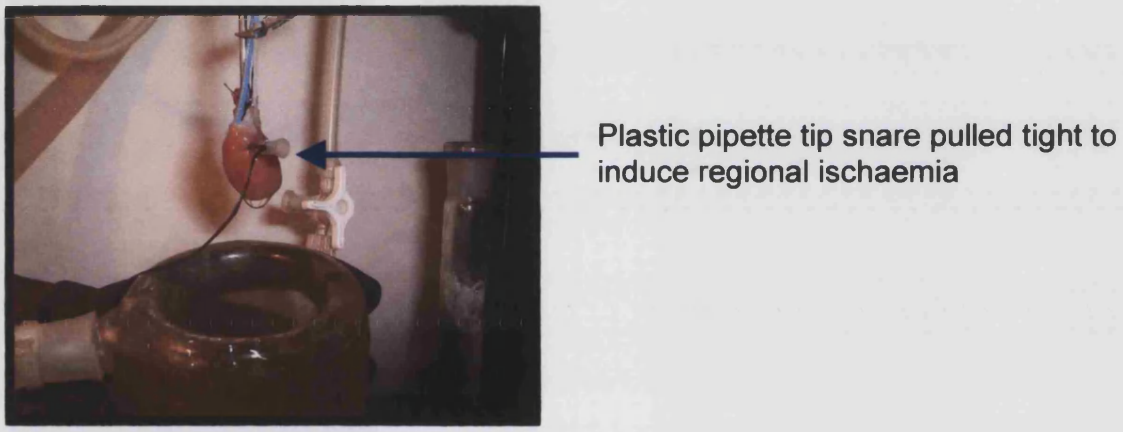
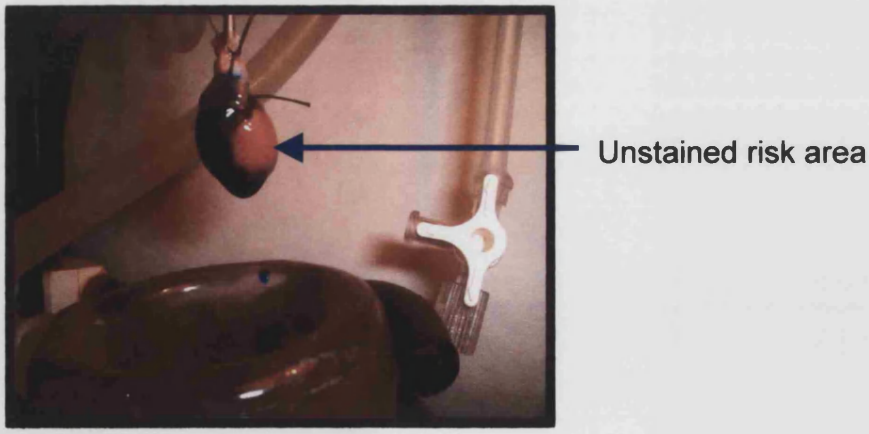


Figure 3.4: ***Delineation of the Risk Zone of the Isolated Perfused Rat Heart:*** with the suture tied-off, the perfused Evan's blue stains the non-risk area blue, leaving the risk area unstained.



3.2.2 Determination of Infarct Size

Myocardial Infarct size was determined after 120 minutes reperfusion, using the triphenyltetrazolium technique, which has been used to quantify infarct size in many experimental models.([868;869])

At the end of the 120 minute reperfusion period, the snare was pulled tight and the heart was slowly perfused with saline solution containing 0.25% Evans blue (Sigma Chemicals, Poole, Dorset), to delineate the non-ischaemic zone of the myocardium as a dark blue area (figure 3.4 depicts a heart perfused with Evans blue). After 1 to 4 hours at -20 °C, the hearts were sliced into 5 x 2mm-thick transverse sections from apex to base, and incubated in triphenyltetrazolium chloride (Sigma Chemicals, Poole, Dorset) solution (1% in phosphate buffer, pH 7.4) at 37°C for 15 minutes.

Measuring infarct size using tetrazolium staining depends on the ability of dehydrogenase enzymes and cofactors present in viable myocardial tissue reacting with tetrazolium salts and forming the red formazan pigment.([870]) Dehydrogenase enzymes and cofactors are washed out during the phase of reperfusion from the infarcted tissue and therefore do not stain with the red formazan pigment and appear as white areas devoid of pigment. In the isolated crystalloid-perfused rat heart, it is essential to reperfuse for at least 2 hours to ensure adequate wash-out of dehydrogenase enzymes and cofactors. Inadequate wash-out of dehydrogenase enzymes and cofactors from use of a shorter reperfusion times will underestimate the infarct size.([868])

The tissue slices were then put in 10% formalin (BDH Laboratory Supplies, Poole, UK) for 24 hours, to make the infarcted area easier to distinguish. In the risk zone the viable tissue was stained red and the infarcted tissue appeared pale (figure 3.5 depicts heart slices stained with tetrazolium). The stained slices were then placed between two clear Perspex plates which were held together with spring clamps, and were then traced onto clear acetate and with the use of a computerised planimetry package (Summa Sketch III, Summagraphics, Seymour, CT, USA), the percentage of infarcted tissue within the volume of myocardium at risk was calculated and expressed as infarct-risk volume ratio (I/R%) (figure 3.6 depicts the planimetered heart slices).

Figure 3.5: ***Tetrazolium-Stained Heart Slices***: taken from (a) control hearts (35 minutes of left main coronary artery occlusion followed by 120 minutes of reperfusion) and (b) ischaemic preconditioned (IPC) hearts (which underwent the same ischaemia-reperfusion protocol, preceded by an IPC protocol comprising two five minutes coronary occlusions with an intervening 10 minutes reperfusion). The slices demonstrate areas of infarction (white), non-infarcted risk area (red) and non-risk area (blue). Note the smaller infarct area in preconditioned hearts vs the control hearts.

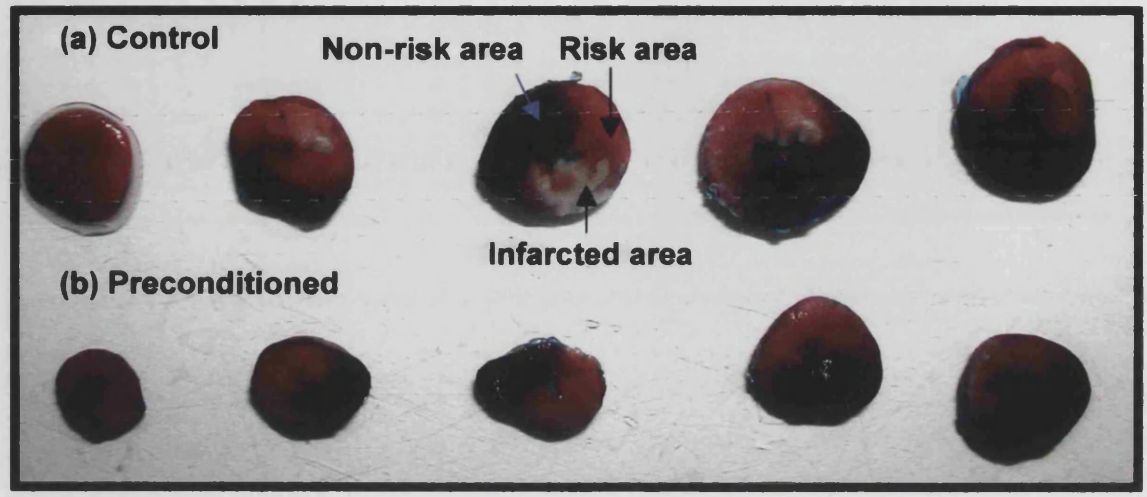
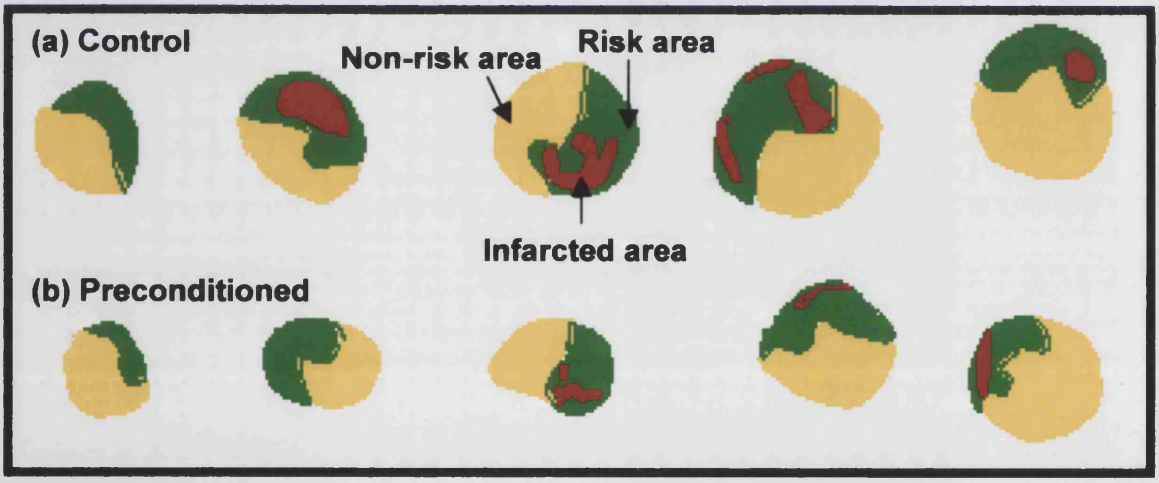


Figure 3.6: ***Planimetred Heart Slices***: taken from (a) control and (b) ischaemic preconditioned hearts stained depicting infarcted areas (red), non-infarcted risk area (green) and non-risk area (yellow).



3.2.3 Exclusion Criteria

Hearts were excluded from the study if after the 30 minutes stabilisation period, the rate pressure product (RPP, heart rate multiplied by left ventricular developed pressure) was less than 17 mmHg/min or the coronary flow rate was <10 ml/min. Hearts were also excluded if the risk volume was either less than 0.3 cm^3 or greater than 0.7cm^3.

3.3 Preparation of Adult Rat Mitochondria

The centrifuge was pre-cooled to 4°C by spinning with no samples for 15 minutes at 5800 *g*. Rats were anaesthetised with sodium pentobarbital (55 mg/kg intra-peritoneally, Sagatal-Rhone Merieux), and were given heparin sodium (300 IU intra-peritoneally, Multiparin CP Pharmaceuticals Ltd.) to prevent thrombus formation in the heart and vessels. When the rat was sufficiently anaesthetised, indicated by the loss of the pedal withdrawal reflex, a trans-abdominal incision was made and the thoracic cavity exposed by a bilateral incision along the lower rib margin. The anterior thoracic cage was then reflected superiorly, and the heart was gently held between the fingers and excised.

Immediately after excision, the heart was immersed in cold perfusion solution (at 4°C to limit ischaemic injury) and then mounted on an adapted constant flow (at 14 ml/min) non-recirculating Langendorff-perfusion apparatus, and perfused for 5 minutes with modified Krebs-Henseleit buffer containing (in mmol/l): NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.7, and glucose 12, to displace all the blood.

The heart was then removed from the perfusion apparatus and cut into pieces in a Petri dish and placed into a 50 ml falcon tube containing Isolation Buffer 1 which comprised (in mmol/l): mannitol 210, sucrose 70, Tris/HCl 10, fatty acid-free BSA 0.5 mg/ml, and EGTA 1 (pH 7.3). The heart tissue was then homogenised, using a Polytron model T25 homogeniser (IKA Labortechnik T25, Janke & Kunkel GmbH & Co., Germany), for 15 seconds and the solution was centrifuged at 450 *g* for 5 minutes at 4°C. Following this, 35 ml of the supernatant was removed and transferred to a reinforced centrifuge tube and spun at 5800 *g* for 10 minutes at 4°C. The remaining 15 ml containing the pellet was discarded. The 35 ml supernatant was then decanted and discarded and the pellet was re-suspended in 5 ml of Isolation Buffer 2, which comprised (in mmol/l): mannitol 210, sucrose 70, Tris/HCl 10. The solution was then spun at 5800 *g* for 10 minutes at 4°C. The supernatant was decanted and the pellet was re-suspended in 3 ml of Respiratory Buffer 1 which comprised in mmol/l: sucrose 250, HEPES 10, K_2HPO_4 2. To this, 3ml of Respiratory Buffer 2 was added, which contained in mmol/l: HEPES 10, KCl 80, Mg acetate 2.

After extraction, the mitochondria were kept on ice suspended, in the 6 ml of the combined respiratory buffers (at pH 7.5), and were used within 6 hours. To ensure equal amounts of mitochondria were used for each experiment the protein content of each

mitochondrial sample was determined, and 0.5 mg/ml of mitochondrial was used for the experiment (see section 3.3.1).

The viability of the isolated mitochondrial sample, was tested by determining the mitochondrial membrane potential (an indication of the extent of mitochondrial respiratory coupling), using flow cytometrical analysis (see section 3.4).

3.3.1 Protein Quantification

The Bicinchoninic acid-based (BCA™) protein assay reagent system (Pierce, Rockford, USA) was used to estimate the protein concentration of the mitochondrial samples. Duplicate mitochondrial samples were incubated for 30 minutes at 37°C, with a mixture of Cu^{2+} and bicinchoninic acid (BCA), which is originally green in colour, and forms a purple solution on formation of the BCA-Cu^{2+} complex. The optical density of the solution was determined by measuring the absorbance at 562 nm, using a photo-spectrometer (Janway model 6405 UV/Vis, Dunmow, UK). The relationship between the absorbance and protein content is linear over (20-2000 µg/ml), and therefore a standard calibration curve can be obtained by measuring the absorbance for duplicate samples containing increasing concentrations of bovine serum albumin (BSA): 0, 10, 20, 30, 40 µg/µl. Using the standard curve, the protein content (µg/µl) for each mitochondrial sample was then derived from the optical absorbance for that particular sample.

3.4 Flow Cytometry

Flow cytometry is the measurement of cells in a flow system: and applies to instruments which focus light on to cells and record their fluorescence and light scattered by them. Typically, five parameters can be measured: using blue light (generated by the 488-nm argon laser) for excitation, one can record green, orange, and red fluorescence and blue light scattered in a forward direction (forward scatter) and at right angles to the laser beam (side-scatter). Cyto-fluorometric analysis was performed on a Partec flow cytometer (Partec, Münster, Germany) equipped with a 488-nm argon laser (see figure 3.7).

In the present study, the 488-nm laser was used to excite a fluorescent compound within mitochondria which absorbs the light and then emits light at a lower energy and therefore longer wavelength than the exciting light (see figure 3.8). Much of the light directed at the mitochondria is scattered in a forward or sideways direction by the mitochondrial surface (so-called forward and side scatter, see figure 3.8 and 3.9). However, some of the light is absorbed by the fluorescent compound within the cell and is emitted at a longer wave-length, thereby enabling its differentiation from the light scatter. Measurements are made as the mitochondrial sample flows through the instrument. The mitochondria are reproducibly delivered to the measuring point, using hydrodynamic focusing, which involves introducing a slow stream of mitochondria into a quickly moving carrier fluid (which constrains the mitochondria to the centre of the tube). The emitted light is directed to the appropriate detector, directed through the use of dichroic and band-pass filters. Dichroic filters are selective mirrors which allow the transmission of long wavelengths while reflecting short wavelengths (see figure 3.8). Band-pass filters allow light of a specific wavelength, or narrow band of wavelengths, to pass through.

To determine the viability of the isolated mitochondrial sample, a 2 ml 0.5 mg/ml sample was incubated with 200 nmol/l of the fluorometric probe, tetramethyl rhodamine methyl ester (TMRM, Molecular Probes Inc., Leiden, The Netherlands), for 2 minutes at room temperature. TMRM is a cationic probe which reversibly accumulates in the mitochondria according to the negative mitochondrial membrane potential with a Nernstian distribution.([845]) Using the flow cytometer, the TMRM fluorescence (an indicator of mitochondrial membrane potential) was measured, both before and after the addition of 1 μmol/l of the ionophore, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma Chemicals, Poole, Dorset), which by uncoupling mitochondrial respiration should result in a reduction in TMRM fluorescence

(indicating collapse of the mitochondrial membrane potential). Mitochondrial samples were excluded if they did not produce these changes in mitochondrial membrane potential, indicating that they were not respiratory coupled.

For TMRM fluorescence, the signal was analysed in the FL2 channel equipped with a band-pass filter at 580 nm; the photo-multiplier value of the detector was 631 V. The flow cytometer counts the number of mitochondria (depicted as 'Counts' on the Y scale) at a particular fluorescence or light intensity on the logarithmic X scale (see figure 3.9). Arithmetic mean values of the median fluorescence intensities were determined for each sample for graphic representation.

Figure 3.7: Flow Cytometer

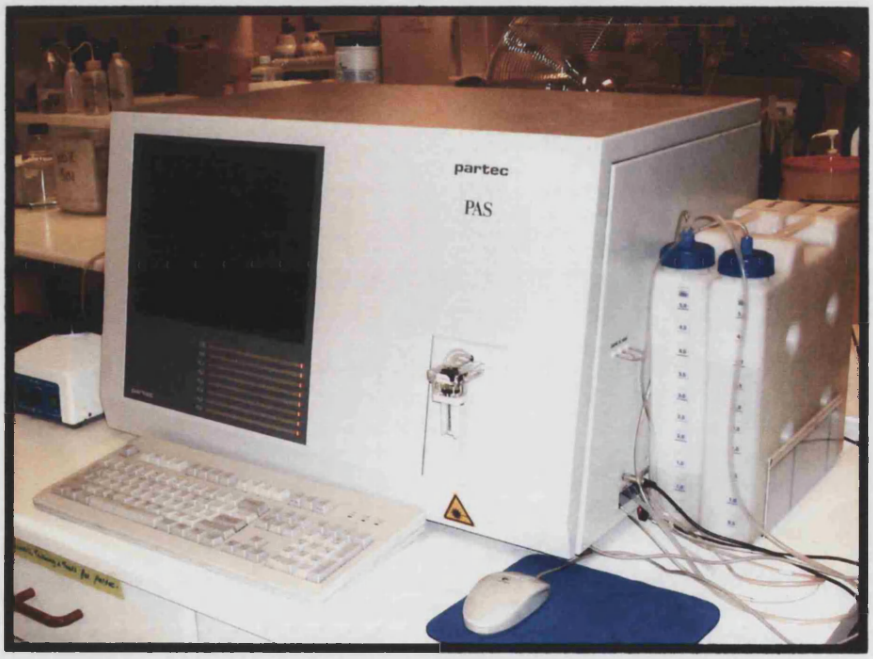


Figure 3.8: Basic Principles of Flow Cytometry: Light from the 488-nm Argon laser is directed at the downward moving mitochondrial sample. Light is absorbed by the fluorescent probe contained within the mitochondria, and is emitted at various lower energy longer wavelengths, which are then directed by means of the dichroic and band-pass filters to the appropriate detector: FL1 for mitochondrial calcein fluorescence and FL2 for mitochondrial TMRM fluorescence. Light scattered on the mitochondrial surface is detected as forward and side scatter at the same wave-length.

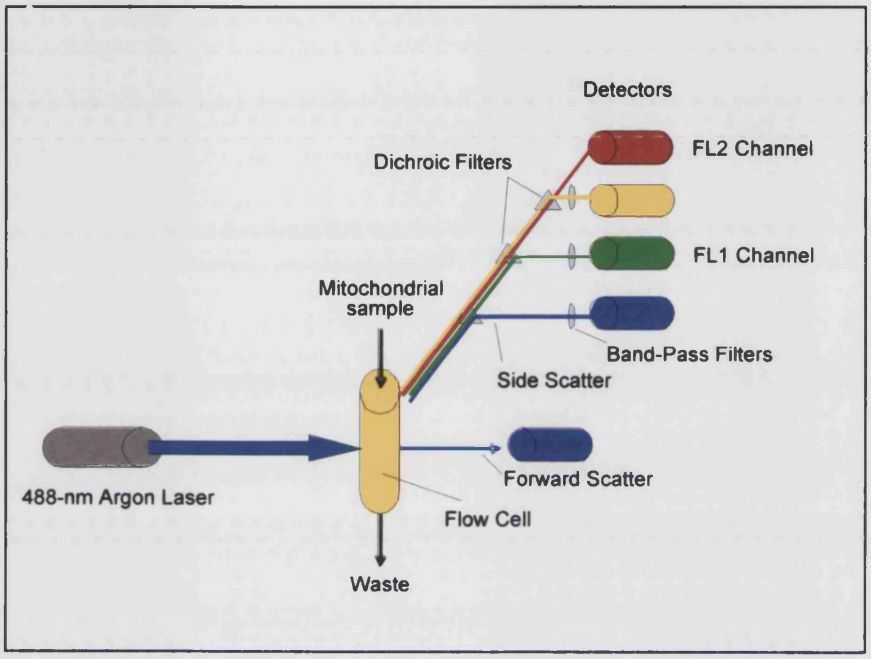
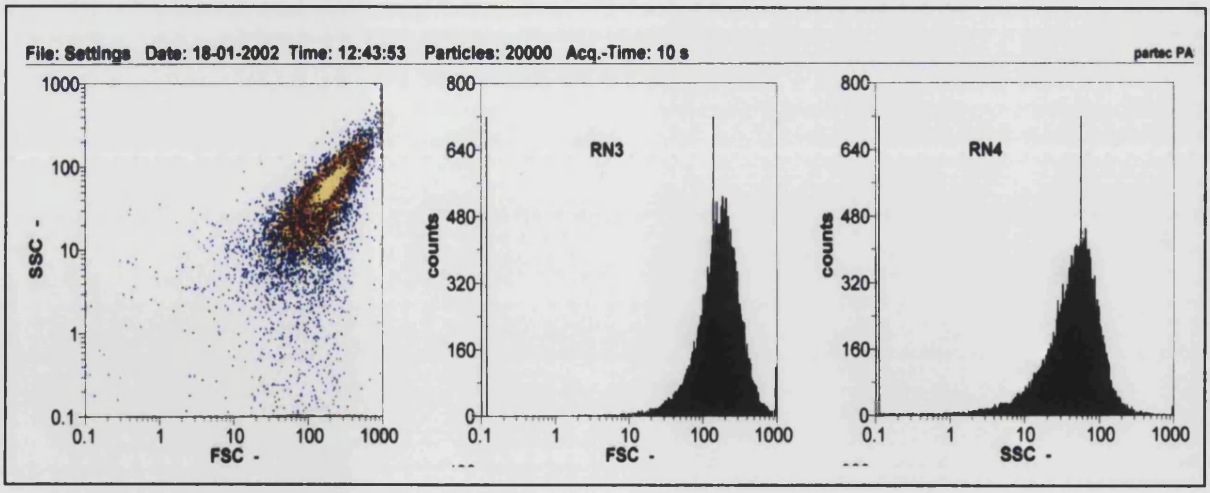


Figure 3.9: Representative Flow Cytometric Profile of Unstained Mitochondria: Light scattered on the mitochondrial surface is detected as forward (FSC) and side scatter (SSC) at the same wave-length, demonstrating a distinct mitochondrial population.



3.5 Preparation of Adult Rat Myocytes

Isolated ventricular myocytes provide a uniform population of a cell type free from neuro-hormonal influence in an environment which can be controlled. Rats were anaesthetised with sodium pentobarbital (55 mg/kg intra-peritoneally, Sagatal-Rhone Merieux), and were given heparin sodium (300 IU intra-peritoneally, Multiparin CP Pharmaceuticals Ltd.) to prevent thrombus formation in the heart and vessels. When the rat was sufficiently anaesthetised, indicated by the loss of the pedal withdrawal reflex, a trans-abdominal incision was made and the thoracic cavity exposed by a bilateral incision along the lower rib margin. The anterior thoracic cage was then reflected superiorly, and the heart was gently held between the fingers and excised. Immediately after excision, the heart was immersed in cold perfusion solution (at 4°C to limit ischaemic injury) and then mounted on an adapted constant flow (at 14 ml/min) non-recirculating Langendorff-perfusion apparatus (figure 3.7).

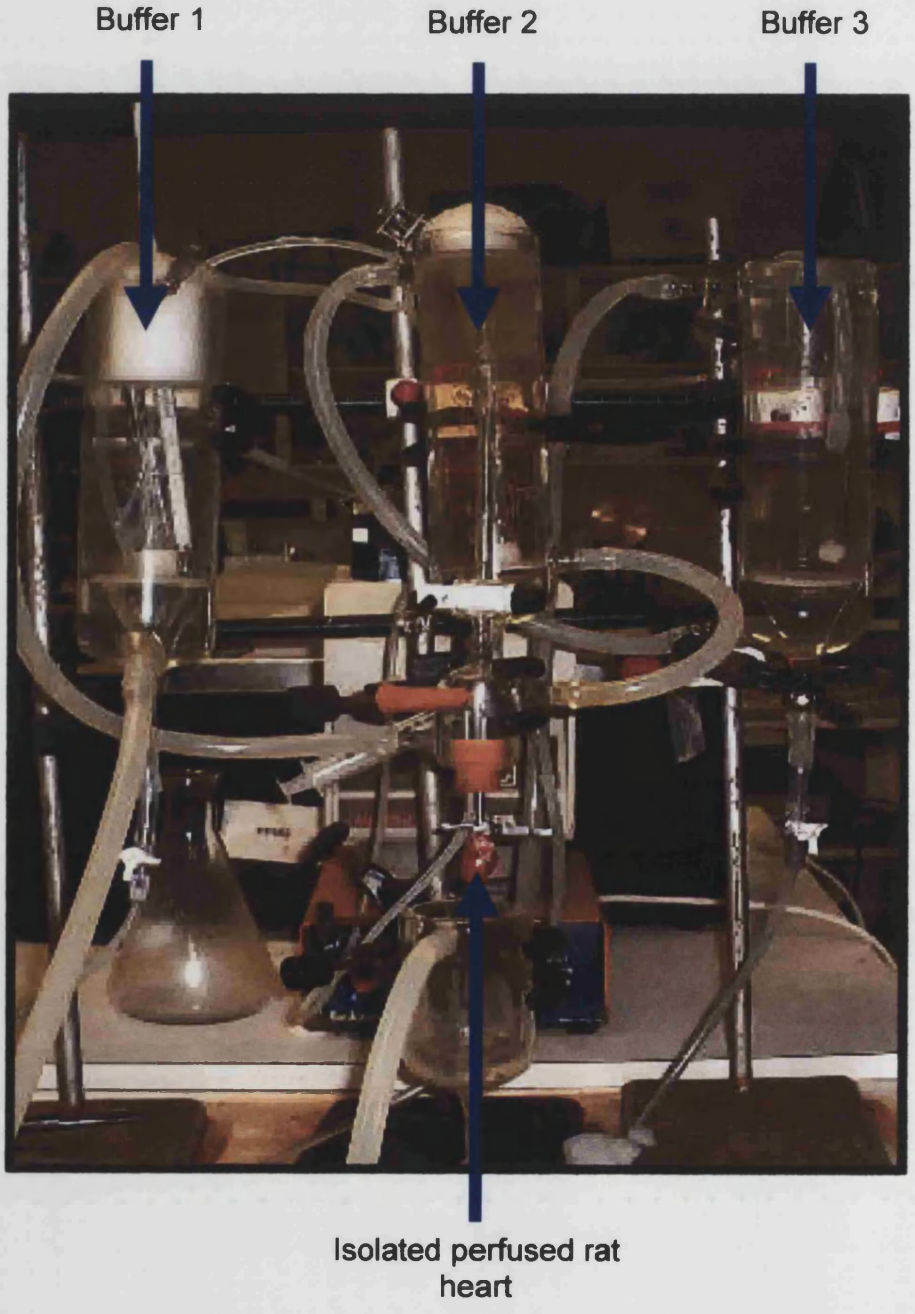
All buffers were made from autoclaved distilled water and were based on a modified calcium-free Krebs-Ringer's-HEPES (KRH) buffer (in mmol/l): 116.0 NaCl, 5.4 KCl, 0.4 $MgSO_4$, 20.0 HEPES, 0.9 Na_2HPO_4, 5.6 glucose (pH 7.4). All solutions were filter-sterilised prior to use. The perfusate was bubbled with 100% O_2 and maintained at 37°C using a thermostatically-controlled water-jacketed system in which all glass reservoirs, and the heart perfusion chamber are surrounded by rapidly flowing warmed water at 37.0-37.5°C, using a water circulator.

The heart was initially perfused with Buffer 1 comprising KRH buffer containing 1 mg/ml BSA and 3.3 μmol/l EGTA (figure 3.7). After 5 min the heart was perfused with Buffer 2 comprising KRH buffer containing 0.75 mg/ml collagenase (Type II LS004176, Worthington Biochemical Corporation, Lakewood, NJ), and 25 μmol/l calcium for 10-15 min (figure 3.7). The heart was finally perfused with Buffer 3 comprising KRH buffer containing 50 μmol/l calcium for 5 minutes (figure 3.7). Following perfusion the heart was removed from the Langendorff apparatus and the atria trimmed away. The ventricles were then carefully cut into strips and placed in a Petri-dish with collagenase for 10 minutes, and the Petri-dish was gently agitated at 37°C. Harvested cells were then filtered through a nylon mesh and washed with restoration buffer comprising KRH buffer + (in mmol/l) 0.5 Na pyruvate, 5.0 taurine, 2.0 carnitine, 1.0 creatine + 75 μmol/l calcium + 10 mg/ml BSA, using low speed centrifugation (1000 rpm for 30 seconds) to sediment the cells. The remaining undigested heart tissue underwent a further 20

minute collagenase digestion, which usually resulted in complete dispersal of ventricular cells. Again these cells were washed in restoration buffer and these were then added to the cells from the first collagenase digestion. The calcium concentration was increased in a step-wise manner to 1.25 mmol/l, by adding 5 aliquots of 235 µmol/l calcium, with one added every 15 minutes, in order to render the cells calcium-tolerant.

After isolation, cell viability was assessed using light microscopy and routinely averaged 80-90%. Isolations yielding less than 60-70% viable cells were excluded.

Figure 3.10: ***Langendorff Perfusion Apparatus for Preparing Adult Rat Myocytes:*** the isolated rat heart is perfused in sequence with the 3 buffers.



3.6 Preparing Myocytes for Confocal Microscopy

For confocal microscopy, the cells had to be seeded onto 25 mm diameter round glass cover-slips. All the following procedures were conducted in a sterile positive-pressure Microbiological Safety Cabinet (Walker Safety Cabinets Ltd, Derbyshire, UK). Before use, these cover-slips were sterilised with 100% ethanol and were washed in sterilised phosphate buffered saline (PBS). Once the cover-slips were dry, 200 µl of laminin (1mg/ml diluted in 30 ml of distilled water, Sigma Chemicals, Poole, Dorset), was pipetted onto the centre of the cover-slip and left to dry for 1-1½ hours. 500 µl of the cell suspension was then carefully placed on the laminin residue in the centre of the cover-slip. The cells were left to settle and seed to the cover-slips for 1½-2 hours in an incubator (CO_2 Incubator, CO28IR, New Brunswick Scientific, USA) at 37°C in an atmosphere of 95% air/ 5% CO_2. After adequate seeding, the restoration buffer was carefully replaced with M-199 medium (M7653, Sigma Chemicals, Poole, Dorset) containing 10% foetal calf serum (Sigma Cell Chemicals, Poole, Dorset) and 1% penicillin-streptomycin (Sigma Chemicals, Poole, Dorset), and the cells were incubated overnight at 37°C in an atmosphere of 95% air/ 5% CO_2. Next day the cells were washed with restoration buffer and kept in the incubator until use.

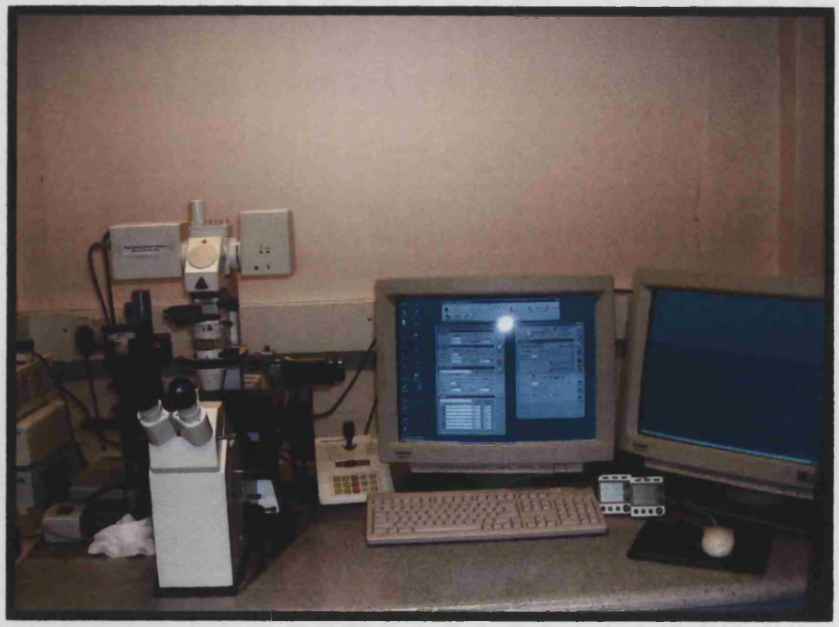
3.7 Confocal Microscopy

All confocal imaging and analysis was conducted in the Mitochondrial Biology Group, the department of Physiology, University College London in collaboration with Professor Michael Duchen. The 25 mm round cover-slip containing the seeded myocytes was placed in a custom-made chamber with 1 ml of restoration buffer and mounted on the stage of a Zeiss 510 CLSM confocal microscope equipped with x40 oil immersion, quartz objective lens (NA 1.3).

For measuring tetramethylrhodamine methyl ester (TMRM) fluorescence, the cells were illuminated using the 543 nm emission line of a henna laser. The fluorescence of TMRM was collected using a 585 nm long pass filter. For measuring calcein fluorescence, the cells were illuminated using the 488 nm emission line of an Argon laser. The fluorescence of calcein was collected using a 505 nm long pass filter.

Images were analysed using the Zeiss software (LSM 2.8) and also using Lucida (Kinetic Imaging, Wirral) to measure changes in mean and SD of the signals with time.

Figure 3.11: Confocal Microscope With Camera Attached



3.8 Western Blotting

3.8.1 Collecting Tissue Samples

Hearts were isolated from anaesthetised male Sprague-Dawley rats and mounted on the Langendorff-apparatus and subjected to the relevant ischaemia-reperfusion protocols set out in Chapter 6. At time points determined by the relevant protocols, coronary flow was terminated and the snare, that was used to induce regional ischaemia (see section 3.2.1), was pulled tight and the heart was slowly perfused with saline solution containing 0.25% Evans blue (Sigma Chemicals, Poole, Dorset), to delineate the ischaemic risk zone of the myocardium as the unstained area (figure 3.4). The ischaemic risk zone was excised from the rest of the heart, and immediately snap-frozen in liquid nitrogen using pre-cooled tongs. The heart sample was then broken into small pieces and stored at -70°C for Western blot analysis.

3.8.2 Protein Extraction

For each collected heart sample (see section 3.8.1), approximately 50 mg of frozen heart tissue was used. The tissue was homogenised, using a Polytron model T25 homogeniser (IKA Labortechnik T25, Janke & Kunkel GmbH & Co., Germany), on ice in 250 µl of *suspension buffer*, which comprised (in mmol/l): NaCl 100, TRIS 10 (pH 7.6), EDTA 1 (pH 8.0), sodium pyrophosphate 2, sodium fluoride 2, β-glycerophosphate 2, phenyl methyl sulphonyl fluoride (PMSF) 0.1 µg/ml, and 1 µg/ml each of aprotonin, leupeptin, trypsin inhibitor and protease inhibitor. This part of the procedure lyses the cells and releases proteins. The latter constituents of the sample buffer are protease inhibitors which prevent protein degradation of the sample.

The homogenised samples were then centrifuged at 10,000 rpm for 10 minutes at 4°C and the pellet (containing cellular debris) discarded. The protein content of the supernatant from each protein sample was determined using the Bicinchoninic acid-based (BCA™) protein assay reagent system (Pierce, Rockford, USA)(see section 3.3.1). The protein sample was then diluted in 2x *sample buffer*, comprising (in mmol/l): Tris 100 (pH 6.8), dithiothreitol (DTT) 200, sodium dodecylsulphate (SDS) 2%, bromophenol blue 0.2%, and glycerol 20%) and subsequently heated for 10 minutes at 100°C, and then stored at -70°C for later analysis.

3.8.3 Preparing the Gel and Electrophoresis

For the present study, SDS-PAGE gels were used. The gel was made between two carefully cleaned glass plates separated by spacers and combs, and the edges were sealed with agarose gel. The gel consisted of:

(1) *the 5% stacking gel* (comprising 7.0 ml de-ionised H_2O, 3 ml stacking gel base [comprising 0.5 M TRIS, 0.4% SDS in de-ionised H_2O, pH 6.8], 2 ml 30% acrylamide, 20 µl 8% bromophenol blue, 24 µl TEMED and 120 µl 10% ammonium persulphate) and

(2) *the 12.5% running gel* (comprising 12 ml de-ionised H_2O, 9 ml stacking gel base [comprising 1.5M TRIS, 0.4% SDS in de-ionised H_2O, pH 8.8], 15 ml 30% acrylamide, 40 µl TEMED and 200 µl 10% ammonium persulphate).

The SDS is the denaturing agent that binds proteins so they become negatively charge and therefore separate on the basis of size alone and not on the intrinsic protein charge. The APS is a polymerisation catalyst required for gel formation. The TEMED is also a polymerisation catalyst, which catalyses the formation of persulphate free radicals from the APS, which in turn initiates polymerisation.

For each lane of the stacking gel, 30 µg of protein was loaded into each well. A molecular weight Rainbow marker was also loaded (10 µl) onto the gel. The gel was then placed in a vertical electrophoresis system containing running buffer (comprising glycine 14.4 g/l, SDS 1.0 g/l, Tris 3.0, distilled H_2O 1 litre) and the gel was allowed to run for 4-5 hours at 125 V.

3.8.4 Protein Transfer

After electrophoresis, the gel was mounted in a tank transfer system containing transfer buffer (200 ml methanol, 700 ml distilled H_2O, 100 ml of *transfer buffer* [glycine 14.4 g/l, Tris 3.0 g/l, distilled H_2O 1 litre]). The gel was carefully placed on a Hybond ECL nitrocellulose membrane (Amersham, UK), cut to the same shape as the gel. The gel and membrane were then placed between two pieces of Whatman paper, and any air bubbles were removed. The proteins were then allowed to transfer overnight (12-16 hours) at 140 mA. The gels were stained with Coomassie blue to confirm adequate transfer of proteins. After Western blotting, the membrane was then removed and adequate transfer of proteins was further confirmed by Ponceau Red staining of the membranes (Sigma Chemicals, Poole, UK).

3.8.5 *Antibody Probing*

The membrane was then placed on a rocking platform and washed for 5 minutes in *PBS-Tween* (comprising Na_2HPO_4 1.6 g/l, NaCl 8.0 g/l, distilled H_2O 1 litre, Tween-20 500 µl, pH 7.2), before being placed in *blocking buffer* (PBS-Tween with 5% Marvel) for 2 hours. The blocking buffer blocks potential binding sites on the membrane, in order to reduce non-specific binding of the antibodies onto the membrane. Following this, the membrane was washed 3 times for 5 minutes each time in PBS-Tween, and then placed in the *primary antibody solution* (comprising PBS-Tween with the antibody at 1:1000 dilution+ 3% BSA) for 3 hours.

The membrane was then washed a further three times for 5 minutes each time and then subsequently probed with horse-radish peroxidase-conjugated secondary anti-rabbit antibody solution (comprising PBS-Tween with antibody at 1:2000 dilution in + 5% Marvel) for 1 hour. The membrane was then washed a final three times for 5 minutes each time. The proteins were detected using enhanced chemi-luminescence ECL Western blotting detection reagent and the bands were visualised by exposing the membrane onto Kodak AR film.

3.8.6 *Quantification of Protein Bands*

The developed films were scanned on a flat-bed picture/document scanner, and the digital image was assessed using the National Institutes of Health (NIH) Shareware program, NIH Image (version 1.63). The relative densitometry for individual protein bands was determined by the grey scale technique, using the supplied program 'Gel plotting macro'. The values were corrected if required for equal protein loading as determined by probing for β-actin.

3.9 Statistical Analyses

All results are presented as group means ± standard error of the mean (SEM). For comparison between more than two groups, factorial one way analysis of variance (ANOVA) was employed. Where a significant F-value was obtained, the Fishers protected least significance difference (PLSD) post hoc test was applied for between group comparisons. For analysing data recorded over a period of time, ANOVA for repeated measures was employed, and where significance was found, the Fishers PLSD post hoc test was applied. Results were considered significant when $P \leq 0.05$. All statistical analysis was carried out on a Power Macintosh computer, using Statview statistical software (Version 4.5, Abacus Concepts Inc.).

Chapter Four

THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE AS A MEDIATOR OF LETHAL REPERFUSION INJURY

4.1 Introduction

In section 1.5.5, the role of the mitochondrial permeability transition pore (mPTP) during ischaemia-reperfusion injury was reviewed. Opening of the mPTP in the first few minutes of reperfusion, following an episode of lethal ischaemia, is a critical determinant of cell death in ischaemia-reperfusion. The conditions that prevail during the first few minutes of reperfusion act in concert to mediate the opening of the mPTP in the transition from ischaemia to reperfusion. These conditions which include a high mitochondrial [Ca^{2+}] and [P_i], ATP depletion, oxidative stress, correction of the acidic pH, are known inducing factors for mPTP opening.([7;8;452]) Opening of the mPTP results in both apoptotic and necrotic cell death,([871]) and the inhibition of its opening during ischaemia-reperfusion injury has been demonstrated to confer protection in various experimental models.

A previous study in the isolated perfused rat heart demonstrated that inhibiting mPTP opening using cyclosporin-A (CsA), restored the ATP/ADP ratio and AMP to pre-ischaemic levels and improved the recovery of LV function following a prolonged length of ischaemia.([780]) In myocytes subjected to lethal hypoxia-reoxygenation, the presence of CsA improved cell viability.([779]) Although cyclosporin-A (CsA) is a potent inhibitor of mPTP opening, it also has other actions,([664]) which include inhibiting the activity of the protein phosphatase, calcineurin.([674]) The phosphorylation of the pro-apoptotic protein, BAD, prevents its binding to 14-3-3, which sequesters it from its mitochondrial target, thereby preventing apoptosis.([743]) Being a phosphatase, calcineurin acts to dephosphorylate BAD, thereby enhancing apoptosis. Therefore, by inhibiting calcineurin, CsA may mediate cardio-protection via this anti-apoptotic mechanism rather than inhibiting mPTP opening.

Recently, the novel immunosuppressant, sanglifehrin-A (SfA), has been shown, to also act as a potent inhibitor of mPTP opening.([676]) This drug has been demonstrated to be a more potent inhibitor of mPTP opening than CsA, and in addition SfA does not inhibit calcineurin.([676;677]) When administered prior to the lethal ischaemic period in the isolated perfused heart, SfA was shown to improve recovery of LV function and reduce LDH release.([676]) In addition, these investigators elucidated that the mechanism of action on the mPTP differed between SfA and CsA, with SfA preventing mPTP opening by inhibiting the peptidyl-prolyl cis-

trans isomerase activity of cyclophilin D, whereas CsA is believed to inhibit mPTP opening by preventing the binding of cyclophilin D to the adenine nucleotide translocase.([676])

However, these studies did not examine the effect of inhibiting mPTP opening using CsA or SfA at the crucial time of reperfusion alone, when the mPTP has been demonstrated to open.([470;776;777]) Furthermore, these studies did not examine the effect of inhibiting mPTP opening at the time of reperfusion on lethal reperfusion injury, in terms of necrotic cell death as measured by infarct size. Therefore the aim of the initial study was to determine whether pharmacologically inhibiting mPTP opening *at the time of reperfusion,* protects the heart against lethal reperfusion injury, using infarct size as the measured end-point of necrotic cell death.

4.2 Hypothesis

Opening of the mPTP at the time of reperfusion is a critical determinant of cell death and inhibiting its opening protects the heart against lethal reperfusion injury

In this initial part of the study, we set out to examine the role of the mPTP in mediating the cell death induced by lethal reperfusion injury. We used cyclosporin-A (the archetypal mPTP inhibitor)([664]) to inhibit the mPTP opening that has been demonstrated to occur in the first few minutes of reperfusion.([470;776;777]) In order to exclude any effect that inhibiting calcineurin may have in this setting, we examined the effect of a drug called tacrolimus (FK506), an immunosuppressant which inhibits calcineurin without affecting mPTP opening.([780]) It was important to use two different known inhibitors of mPTP opening to examine the role of the mPTP in mediating lethal reperfusion injury. Therefore, we also investigated the effect of the newly described mPTP inhibitor, sanglifehrin-A, which also does not inhibit calcineurin.([676]) Importantly, we administered these agents ***only at the time*** of reperfusion in order to demonstrate that the opening of the mPTP is an important mediator of lethal reperfusion injury. We used an isolated Langendorff-perfused rat heart model of ischaemia-reperfusion to demonstrate the effect of pharmacological inhibition of mPTP opening at the time of reperfusion on the cell death induced by lethal reperfusion injury, using infarct size as the measured end-point.

4.3 Aim (1)

To determine whether pharmacologically inhibiting mPTP opening at the time of reperfusion protects the heart against lethal reperfusion injury

4.3.1 Materials

Cyclosporin-A (Sigma Chemicals, Poole, Dorset), and Tacrolimus (FK506, Fujisawa Ltd., Japan) were dissolved in 50% ethanol and added to the Krebs-Henseleit buffer such that the final ethanol concentration was less than 0.005%. Sanglifehrin-A (Novartis Pharma AG, Basel) was dissolved in dimethyl sulphoxide (DMSO, Sigma Chemicals, Poole, Dorset) and added to the Krebs-Henseleit buffer such that the final DMSO concentration was less than 0.01%, which has no effect on infarct size in the Langendorff perfused rat heart. All other reagents were of standard analytical grade.

4.3.2 Isolated Langendorff-Perfused Rat Heart Model

Hearts were excised from male Sprague-Dawley rats and mounted on a Langendorff-perfusion apparatus and subjected to 35 minutes regional ischaemia followed by 120 minutes of reperfusion (see section 3.2.1). At the end of the reperfusion period, the infarct-risk volume ratio was determined using tetrazolium (TTC) staining (see section 3.2.2).

4.3.3 Experimental Protocols for Infarct Studies

The experiment protocols for the infarct studies are presented in Figure 4.1. The hearts were randomly assigned to one of the following treatment groups:

(1) ***Control hearts*** were perfused with the vehicles 0.005% ethanol (n=6), or 0.01% DMSO (n=6) or Krebs-Henseleit buffer alone (n=6) *during* the first 15 minutes of reperfusion;

(2) ***CsA-treatment***: hearts (n=6) were perfused with cyclosporin-A (0.2 μmol/l) *during* the first 15 minutes of reperfusion. This concentration of CsA has been demonstrated to inhibit mPTP opening in the isolated perfused rat heart.([780]) In addition, this concentration provided the most potent inhibition of mPTP opening in adult rat myocytes.([779])

(3) ***Delayed CsA-treatment***: hearts (n=6) were perfused with Kreb's-Henseleit buffer alone for the first 15 minutes of reperfusion followed by cyclosporin-A (0.2 μmol/L) for the next 15 minutes

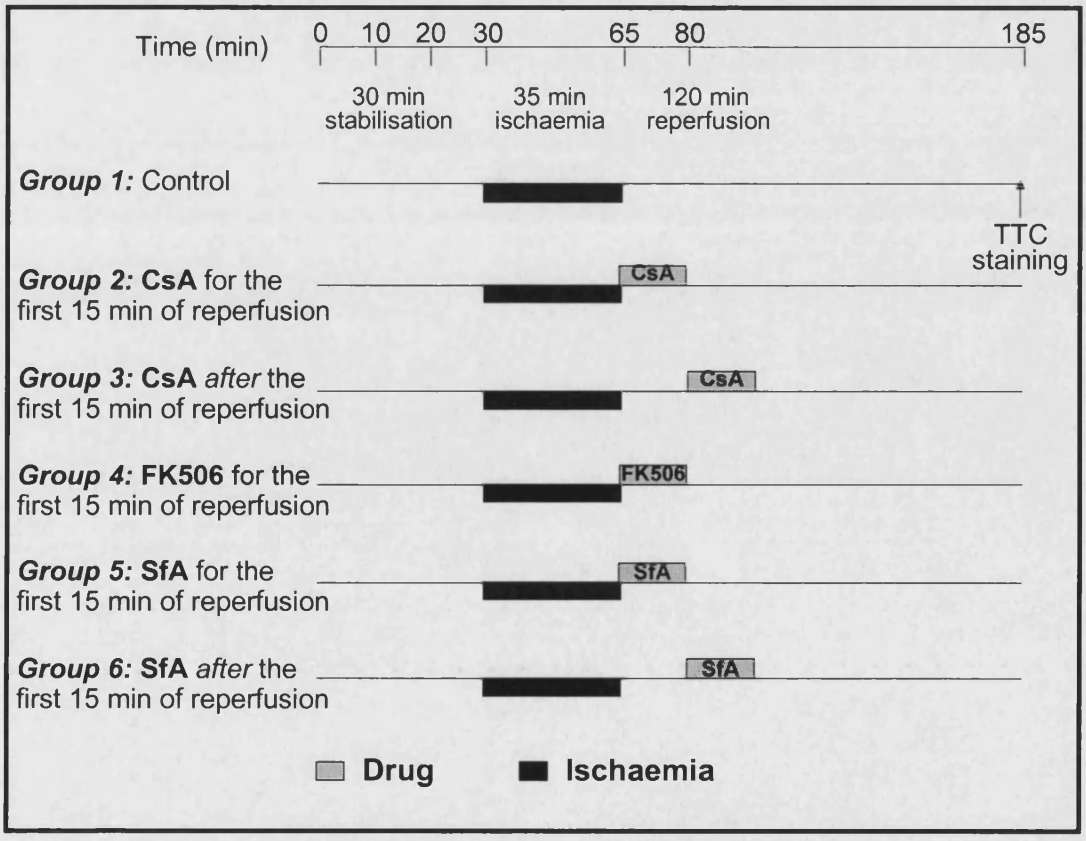
of reperfusion. Therefore, CsA was not given until *after* the first 15 minutes of reperfusion had elapsed;

(4) ***FK506-treatment***: hearts (n=6) were perfused with FK506 (5.0 μmol/l) *during* the first 15 minutes of reperfusion. This concentration of FK506 has been used, to show that the inhibition of the protein phosphatase, calcineurin, was not the mechanism by which CsA induces cardio-protection.([780]) At 5 μmol/l, FK506 has been demonstrated to not inhibit mPTP opening.([704]) At 0.2 μmol/l, CSA causes little or no inhibition of calcineurin activity in the heart, whereas 25 μmol/l of FK506 causes substantial inhibition of calcineurin activity in heart.([872])

(5) ***SfA-treatment***: hearts (n=6) were perfused with sanglifehrin-A (1.0 μmol/l) *during* the first 15 minutes of reperfusion. This concentration of SfA has been demonstrated to give the most potent inhibition of mPTP opening;([676]) and

(6) ***Delayed SfA-treatment***: hearts (n=6) were perfused with Kreb's-Henseleit buffer alone for the first 15 minutes of reperfusion followed by sanglifehrin-A (1.0 μmol/l) for the next 15 minutes of reperfusion. Therefore, SfA was not given until *after* the first 15 minutes of reperfusion had elapsed.

Figure 4.1: ***Experimental Protocols for Investigating the Effect of Inhibiting mPTP Opening on Infarct Size.*** CsA-cyclosporin-A, SfA-sanglifehrin-A, TTC-tetrazolium.



4.3.4 Results

4.3.4.1 Exclusions

We used 52 male Sprague-Dawley rat hearts for the infarct size experiments of which 4 were excluded owing to poor function during stabilisation (see section 3.2.3 for exclusion criteria).

4.3.4.2 Animal and Haemodynamic Data

Animal and heart weights and risk zone volumes were similar in the experimental groups (see table 4.1). Baseline data relating to cardiac function (assessed by the rate-pressure-product, RPP) and coronary flow at the end of the stabilisation period just before the lethal ischaemic period, were similar in the experimental groups (see tables 4.2, 4.3). During regional ischaemia, coronary flow and RPP decreased to a similar extent in both groups. An increase in cardiac function and coronary flow upon reperfusion was indicative of successful re-flow.

The measured end-point, when using the isolated perfused rat heart was the infarct size. To measure left ventricular contractile function accurately, it is necessary to use an isolated working heart model. The method used to measure the left ventricular function in the isolated perfused rat heart is not accurate enough and is not wholly reproducible. In addition, the recovery of left ventricular contractile function (as indicated by the rate pressure product, RPP, which is the left ventricular developed pressure multiplied by the heart rate) following a period of ischaemia-reperfusion, did not always correlate with the reduction in infarct size. The RPP data was used as an indicator that effective regional ischaemia had been induced and that adequate reperfusion had occurred. In addition, any effects of the drug on haemodynamic function could also be monitored.

Table 4.1 Characteristics of Animals in Treatment Groups

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
1. Control	6	359±19	1.90±0.06	0.411±0.017
0.005% ethanol	6	389±38	2.04±0.06	0.405±0.046
2. CsA	6	387±20	1.98±0.07	0.555±0.037
3. Delayed CsA	6	360±12	1.89±0.11	0.490±0.029
4. FK506	6	343±1	1.66±0.04	0.508±0.023
5. SfA	6	339±22	1.72±0.11	0.485±0.046
6. Delayed SfA	6	351±5	2.16±0.07	0.505±0.033

Table 4.2 Rate Pressure Product (x 10^3mmHg/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	29.8±2.0	27.4±2.3	13.3±1.7	13.8±2.2	12.8±2.1	10.1±1.3
0.005% ethanol	37.3±1.0*	33.5±1.0*	15.1±3.1	17.3±4.8	9.7±1.7	6.2±1.4
2. CsA	36.4±1.0*	32.1±2.1	12.5±2.0	18.9±1.4	12.1±0.9	10.8±1.6
3. Delayed-CsA	22.1±0.6*	23.6±2.2	6.4±1.1	9.3±1.0	10.6±2.4	8.2±0.4
4. FK506	33.0±1.1	30.9±0.6	7.6±3.5	13.0±0.8	17.5±0.8	10.3±0.3
5. SfA	26.5±1.9	26.4±1.8	5.8±1.1	14.6±3.0	14.7±0.5	15.4±1.4
6. Delayed SfA	20.4±2.0*	23.0±1.8	8.4±2.2	14.9±2.0	16.1±1.8	11.8±0.6

Table 4.3 Coronary Flow Rate (ml/min)

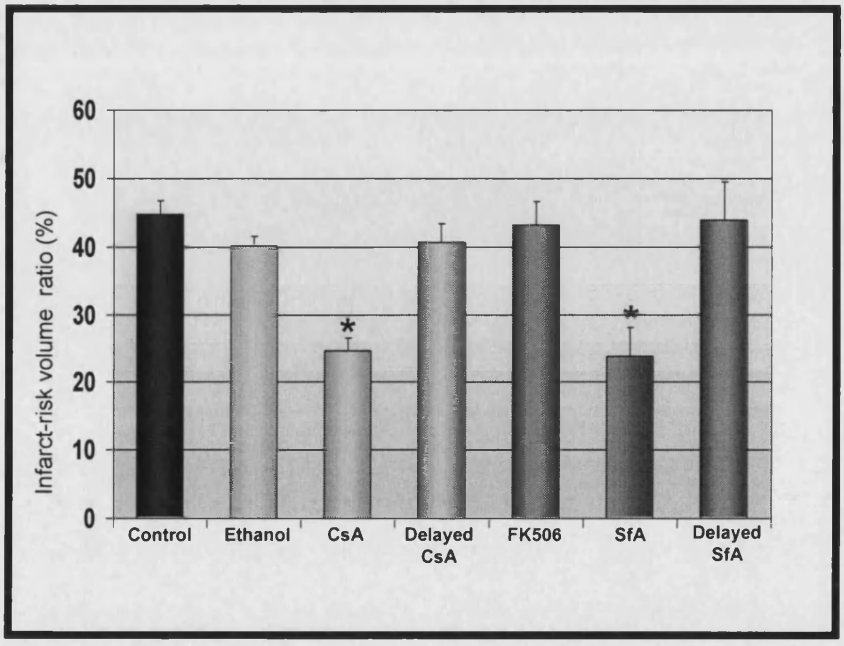
Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	14.6±1.1	12.6±1.2	7.2±0.9	6.0±0.8	8.8±1.4	5.2±0.7
0.005% ethanol	15.7±2.3	15.7±2.3	8.7±1.5	6.7±0.9	6.7±0.7	5.3±0.9
2. CsA	15.8±1.4	13.6±0.7	7.2±0.9	6.0±0.5	7.0±1.0	5.0±0.6
3. Delayed-CsA	19.3±0.9	19.0±0.6*	8.3±0.3	7.3±0.3	10.7±1.3	6.0±1.2
4. FK506	18.3±1.2	18.3±1.2*	8.7±0.7	7.0±0.6	9.0±3.6	6.2±1.9
5. SfA	15.5±1.9	16.0±1.7	8.0±0.4	7.3±0.9	9.8±1.3	8.5±0.6
6. Delayed SfA	21.0±2.4	20.0±2.2*	10±1.2	11±1.9	13.0±1.5	8.3±1.6

Values are mean±SEM. *P<0.05 compared with control.

4.3.4.3 Infarct Size Data

Infarct size is presented as the percentage of tetrazolium-negative tissue in the ischaemic risk zone. The presence of CsA for the first 15 minutes of reperfusion reduced infarct size from 44.7±2.0% in control hearts (40.1±1.4% in ethanol control hearts) to 24.6±1.9% with CsA-treatment (P<0.0001; figure 4.2). However, when CsA was omitted for the first 15 minutes of reperfusion, and only given after 15 minutes of reperfusion had elapsed, there was no influence on infarct size (40.6±2.8% with delayed-CsA vs 44.7±2.0% in control; P=NS; figure 4.2), which suggests that CsA needs to be present during the first 15 minutes of reperfusion to exert its cardio-protective effect. The presence of FK506 at reperfusion did not influence infarct size (43.1±3.5% with FK506-treatment vs 44.7±2.0% in control hearts; P=NS; figure 4.2), suggesting that the inhibition of calcineurin at the time of reperfusion is not cardio-protective. The presence of sanglifehrin-A for the first 15 minutes of reperfusion, reduced infarct size from 44.7±2.0% in control hearts to 23.8±4.2% with SfA-treatment (P<0.001; figure 4.2). However, when SfA was omitted for the first 15 minutes of reperfusion, and only given after 15 minutes of reperfusion had elapsed, there was no influence on infarct size (43.8±5.7% with delayed-SfA given vs 44.7±2.0% in control; P=NS; figure 4.2), which suggests that SfA needs to be present during the first 15 minutes of reperfusion to exert its cardio-protective effect.

Figure 4.2: Inhibiting mPTP Opening at the Time of Reperfusion Protects the Heart Against Lethal Reperfusion Injury. Pharmacologically inhibiting mPTP opening for the first 15 minutes of reperfusion using either cyclosporin-A (CsA) or sanglifehrin-A (SfA) reduced infarct size, although inhibiting mPTP opening *after* the first 15 minutes of reperfusion was not cardio-protective (delayed CsA and delayed SfA). Inhibiting calcineurin for the first 15 minutes of reperfusion, using tacrolimus (FK506) did not influence infarct size.*P<0.001 compared to control.

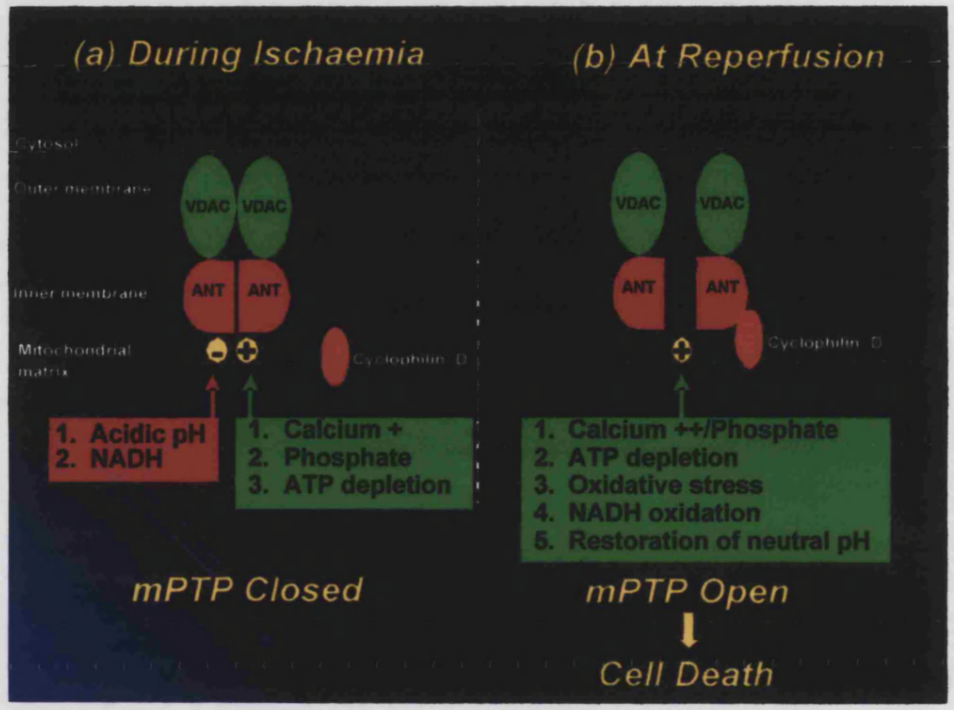


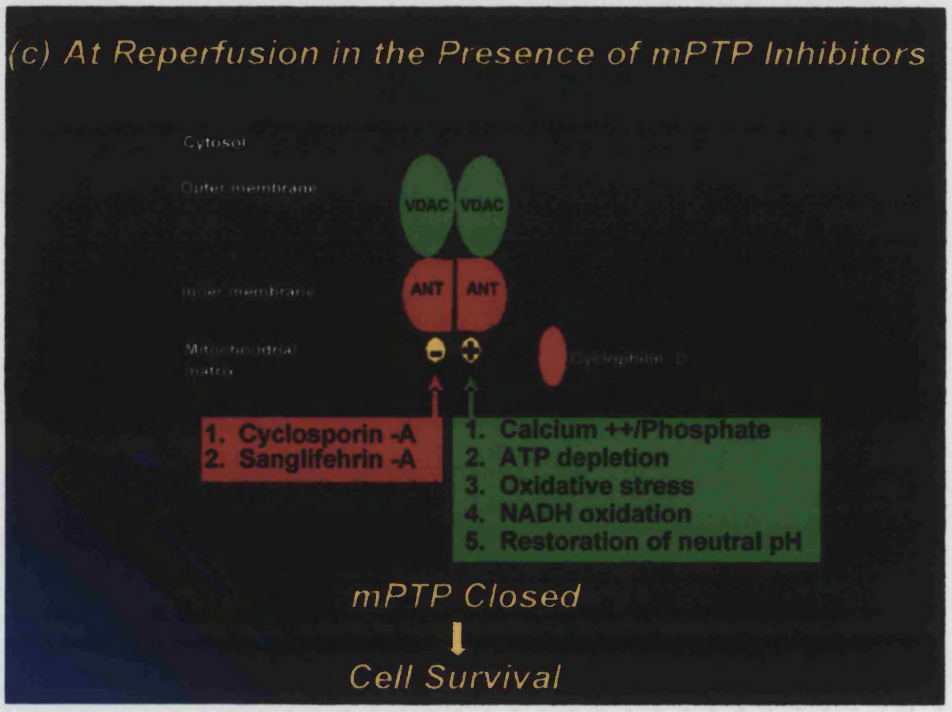
4.3.5 Discussion

In this part of the study we demonstrated that pharmacologically inhibiting mPTP opening for the first 15 minutes of reperfusion, following a lethal period of ischaemia, using either cyclosporin-A (CsA) or sanglifehrin-A (SfA), protected the heart against lethal reperfusion injury, as evidenced by a reduction in infarct size. Importantly, the known mPTP inhibitors were only given at the time of reperfusion, to target the time period when mPTP opening has been demonstrated to occur.([470;776;777])

Furthermore, we demonstrated that targeting the first 15 minutes of reperfusion is crucial to the protective effect of these agents, since the administration of these agents after the first 15 minutes of reperfusion had elapsed, did not protect the heart against lethal reperfusion injury. This suggests that to mediate cardio-protection these drugs have to be present at the immediate onset of reperfusion and for the first 15 minutes of reperfusion, which corresponds to the time-period during which mPTP opening has been demonstrated to occur.([776;777]) Presumably, these drugs are ineffective at reducing infarct size if given after the first 15 minutes of reperfusion because irreversible mPTP opening had already occurred by that stage of reperfusion, in response to the mPTP-inducing factors present at this time (see figure 4.3 a,b). Therefore, providing these agents were administered in a timely manner, they were able to inhibit mPTP opening despite the presence of the mPTP-inducing factors that prevail in the first few minutes of reperfusion (see figure 4.3 a-c).

Figure 4.3 a-c: Modulation of mPTP Opening in Ischaemia-Reperfusion Injury. (a) During ischaemia mPTP opening is inhibited by the acidic matrix pH and NADH despite the presence of Ca^{2+} and P_i and ATP depletion. (b) At reperfusion there is a further rise in mitochondrial Ca^{2+}, a burst of oxidative stress, oxidation of NADH, restoration of physiological pH and cyclophilin D associates with adenine nucleotide translocase (ANT). These factors act in concert to induce mPTP opening at the time of reperfusion, thereby causing cell death. (c) The presence of either cyclosporin-A or sanglifehrin-A at the time of reperfusion inhibits mPTP opening mediating cell survival, despite the presence of the mPTP-inducing factors that prevail during the first few minutes of reperfusion. VDAC-voltage dependent anion channel.





The fact that FK506, when given at the time of reperfusion, provided no protection against lethal reperfusion injury, suggests that the inhibition of calcineurin for the first 15 minutes of reperfusion does not mediate cardio-protection. Therefore, the reduction in infarct size induced by CsA at reperfusion was not due to its effect on calcineurin but was most likely due to its suppression of mPTP opening. In support of this, we also demonstrated protection against lethal reperfusion injury using sanglifehrin-A, which does not inhibit calcineurin.([677;678]) In the previous study by Griffiths and colleagues,([780]) the effect of CsA at the time of reperfusion was previously examined and inhibiting mPTP opening at this time was demonstrated to restore ATP/ADP and AMP to pre-ischaemic levels and improved the recovery of left ventricular contractile function. However, necrotic cell death as measured by infarct size was not determined in this study. Other studies which have investigated the effect of CsA in ischaemia-reperfusion injury have given the drug either before the index ischaemic period or after the onset of ischaemia.([236;800;802]) Interestingly, CsA has been demonstrated to protect the brain when given at the time of reperfusion following a two hour occlusion of the middle cerebral artery, as evidenced by a reduction in infarct size.([796]) Although SfA has been previously investigated in the setting of ischaemia-reperfusion injury, the drug was administered both prior to index global ischaemia and for a further 10 minutes of reperfusion.([676]) Furthermore, in that study the end-points of post-ischaemic recovery of left ventricular function and LDH release were used. As such, it is difficult to interpret whether this agent was only having its effect during the reperfusion phase, given that the drug was administered prior to the lethal ischaemic episode. In addition, necrotic cell death as measured by infarct size was not determined in this previous study.

In conclusion, we have demonstrated that pharmacologically inhibiting mPTP opening during the first few minutes of reperfusion protects the heart against lethal reperfusion injury. From the results of this part of the study, it appears that the opening of the mPTP at the time of reperfusion mediates lethal reperfusion injury, and may be responsible for up to 50% of the infarct size, sustained in this setting. The first few minutes of reperfusion therefore represent a 'window of opportunity' for interventions directed to attenuating lethal reperfusion injury, via inhibition of mPTP opening. In this regard we next examined the newly described phenomenon of 'ischaemic post-conditioning' which has been demonstrated to protect the heart against lethal reperfusion injury,([611]) with the intention of demonstrating that protection occurs via inhibition of mPTP opening.

4.4 Aim (2):

To determine whether ischaemic post-conditioning protects against lethal reperfusion injury by inhibiting mPTP opening at the time of reperfusion

The mechanism associated with the phenomenon of 'ischaemic post conditioning' as first described by Vinten-Johansen in 2001 (and outlined in section 1.4.4), is unknown but based on previous studies, ([612-614]) the controlled gradual reperfusion of ischaemic myocardium may be central to the protective effect. In this regard, we hypothesised that the protection at the time of reperfusion may be mediated via the inhibition of mPTP opening. Opening of the mPTP in the first few minutes of reperfusion in response to the prevailing conditions (which include a high mitochondrial [Ca^{2+}], [P_i], ATP depletion, oxidative stress and a corrected pH), is a critical determinant of cell death in lethal reperfusion injury (see sections 1.5.5 and 4.3). One may envisage that the application of brief intermittent episodes of ischaemia/reperfusion ('ischaemic post conditioning') or the gradual controlled reperfusion employed in the previous studies,([612-614]) may allow the homeostatic mechanisms which govern normal cellular and mitochondrial function to acclimatise to the abrupt metabolic and biochemical conditions associated with the reperfusion phase (listed above), thereby acting to reduce the opening probability of the mPTP at the time of reperfusion.

The data from Downey's laboratory suggest that the MEK1/2-Erk1/2 kinase cascade (a component of the RISK-pathway-see section 1.4.3) may be implicated in the protection associated with 'ischaemic post conditioning'. On this basis, we were therefore interested, to test whether the PI3K-Akt kinase cascade (the other component of the RISK-pathway) is also involved in mediating the protective effect.

We wanted to determine whether the phenomenon of 'ischaemic post-conditioning' could be reproduced in the in vitro Langendorff-perfused rat heart and go on to explore the potential mechanisms involved.

4.4.1 Isolated Langendorff-Perfused Rat Heart Model

Hearts were excised from male Sprague-Dawley rats and mounted on a Langendorff-perfusion apparatus and subjected to 35 minutes regional ischaemia followed by 120 minutes of

reperfusion (see section 3.2.1). At the end of the reperfusion period, the infarct-risk volume ratio was determined using tetrazolium (TTC) staining (see section 3.2.2).

4.4.2. Experimental Protocols for Infarct Studies

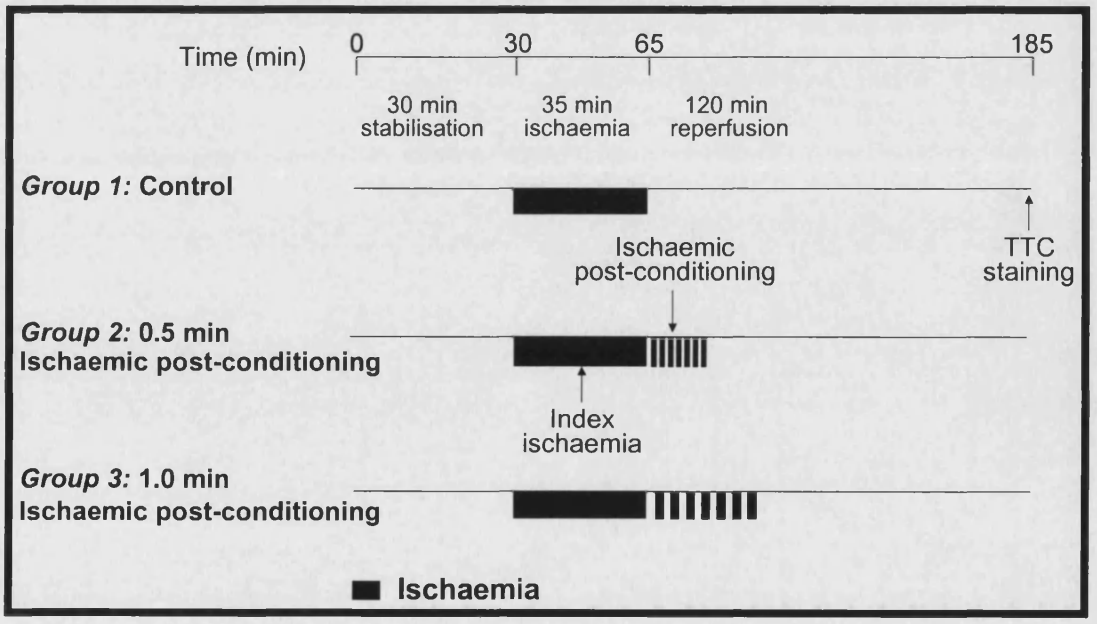
The experiment protocols for the infarct studies are presented in Figure 4.4. The hearts were randomly assigned to one of the following treatment groups:

(1) ***Control hearts***;

(2) ***0.5 minute ischaemic post-conditioning***: hearts (n=6) were subjected to 35 minutes of lethal ischaemia, and then received six alternate episodes of 0.5 minute global ischaemia and reperfusion, and were then reperfused for a further 117 minutes, making the total reperfusion time 120 minutes. This ischaemic post-conditioning protocol had been previously demonstrated to protect the in vivo canine heart.([611])

(3) ***1 minute ischaemic post-conditioning***: hearts (n=6) were subjected to 35 minutes of lethal ischaemia, and then received six alternate episodes of 1 minute global ischaemia and reperfusion, and were then reperfused for a further 114 minutes, making the total reperfusion time 120 minutes.

Figure 4.4: ***Experimental Protocols Investigating Ischaemic Post-Conditioning.*** TTC-tetrazolium.



4.4.3 Results

4.4.3.1 Exclusions

We used 18 male Sprague-Dawley rat hearts for the infarct size experiments of which 0 were excluded (see section 3.2.3 for exclusion criteria).

4.4.3.2 Animal and Haemodynamic Data

Animal and heart weights and risk zone volumes were similar in the experimental groups (see table 4.4). Baseline data relating to cardiac function (assessed by the rate-pressure-product, RPP) and coronary flow before the lethal ischaemic period, were similar in the experimental groups (see tables 4.5, 4.6). During regional ischaemia, coronary flow and RPP decreased to a similar extent in both groups. An increase in cardiac function and coronary flow upon reperfusion was indicative of successful re-flow.

Table 4.4 Characteristics of Animals in Treatment Groups

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
1. Control	6	446±8	1.83±0.06	0.533±0.041
2. 0.5 minute Post-Conditioning	6	324±5	1.37±0.02	0.568±0.038
3. 1 minute Post-Conditioning	6	385±28	1.87±0.20	0.562±0.046

Values are mean±SEM.

Table 4.5 Rate Pressure Product (x 10^3mmHg/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	23.9±3.9	27.4±2.4	17.8±2.3	17.5±5.6	28.1±2.4	13.1±0.1
2. 0.5 minute Post-Conditioning	21.7±2.0	22.7±2.4	10.3±2.7	14.7±4.3	21.0±1.0	13.0±1.0
3. 1 minute Post-Conditioning	27.9±2.7	28.4±2.2	13.2±2.1	10.8±2.5	19.0±0.9	14.6±0.6

Values are mean±SEM.

Table 4.6 Coronary Flow Rate (ml/min)

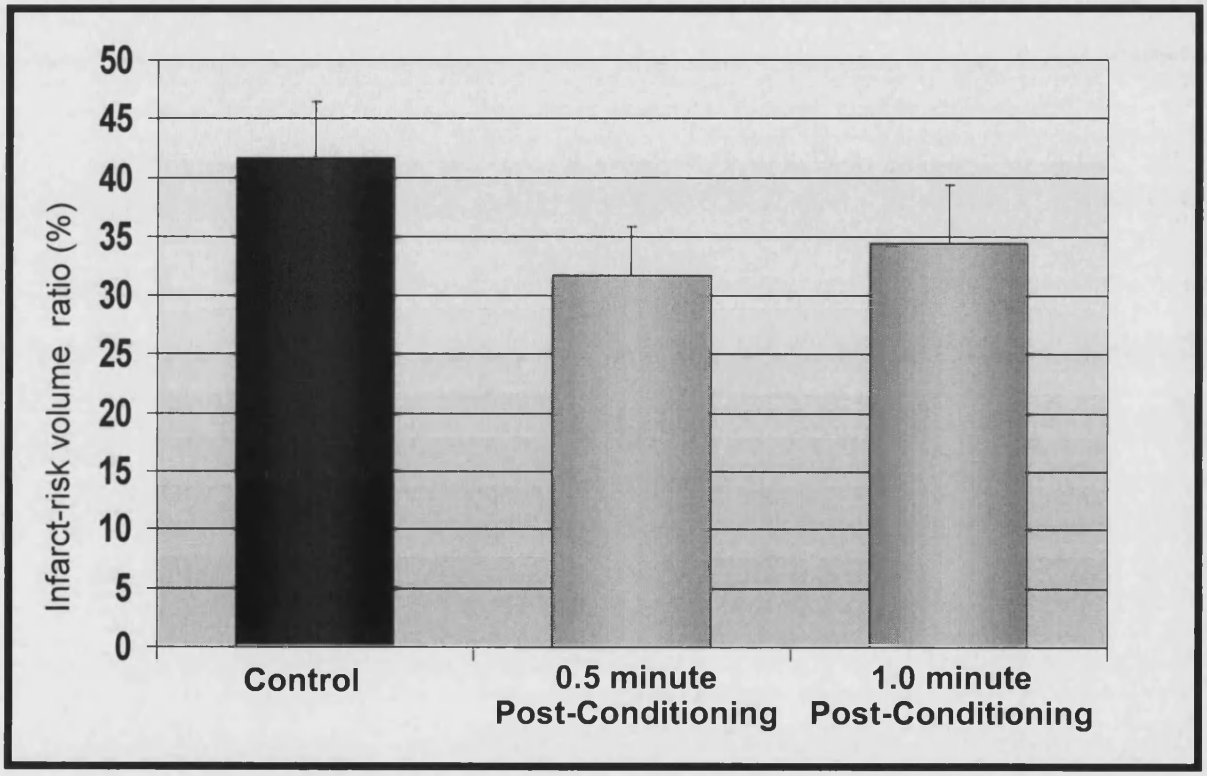
Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	16.0±1.0	16.0±0.6	7.8±0.9	7.0±0.6	12.5±3.5	7.8±1.4
2. 0.5 minute Post-Conditioning	17.5±1.3	18.0±3.0	6.0±1.2	8.7±2.9	10.5±1.0	5.3±0.3
3. 1 minute Post-Conditioning	17.2±1.1	17.2±1.3	6.8±0.6	6.2±0.8	10.8±1.9	7.0±0.6

Values are mean±SEM.

4.4.4.3 Infarct Size Data

Infarct size is presented as the percentage of tetrazolium-negative tissue in the ischaemic risk zone. The 0.5 minute and the 1.0 minute post-conditioning protocols did not reduce infarct size (41.7±4.8% in control hearts vs 31.7±4.1% with 0.5 minute post-conditioning and 34.4±5.6% with 1.0 minute post-conditioning; P=NS; figure 4.5).

Figure 4.5: The Effect of 'Ischaemic Post-Conditioning' on Infarct Size. 'Ischaemic post-conditioning' did not reduce infarct size when compared to control hearts. (Values are mean±SEM).



4.4.4 Discussion

In this part of the study we demonstrated that in the Langendorff-perfused rat heart 'ischaemic post-conditioning' with six episodes of either 1 minute alternate ischaemia/reperfusion periods or 30 second alternate ischaemia/reperfusion periods produced a non-significant reduction in infarct size. The 30 second 'ischaemic post-conditioning' protocol was used initially, based on that used by Zhao and colleagues in the in vivo canine model of ischaemia-reperfusion injury.([611]) After determining that this protocol did not provide significant protection, we used a 1 minute 'ischaemic post-conditioning' protocol, but this produced even less protection. Based on these findings, we did not perform any further investigation of the phenomenon of 'ischaemic post-conditioning'.

The possible reasons for why we did not observe protection against lethal reperfusion injury with 'ischaemic post-conditioning' include:

(1) Difference in species and model used-most of the studies that demonstrated this protective phenomenon used in vivo models of ischaemia-reperfusion injury in rabbit and dog.([611;615]) The fact that Zhao and colleagues observed attenuation in neutrophil accumulation in 'ischaemic post-conditionioned' hearts may suggest the requirement of systemic factors for protection, which would not be present in our in vitro model of ischaemia-reperfusion in the rat. Against this argument, however, is the fact that Vinten-Johansen recently demonstrated 'ischaemic post-conditioning' in the in vivo rat model([616]) as well as in isolated myocytes,([617]) suggesting that the mechanism of protection may be via a direct effect on the myocyte.

(2) The 30 second and 1 minute 'ischaemic post-conditioning' protocols used in our study may have been too long. In the in vivo rat model of 'ischaemic post-conditioning', three times 10 second alternate episodes of ischaemia/reperfusion have now been demonstrated to induce protection.([616]) Interestingly, the extent of protection was not so marked in the rat in vivo model, with a reduction infarct size from 52±3% in control to 40±2%, which compares favourably to the non-significant reduction in infarct size we observed in the isolated rat heart from 42±5% in control hearts to 32±4%. This level of protection observed in the rat heart is in contrast to the large reductions in infarct size observed in the in vivo canine and rabbit models of 'ischaemic post-conditioning', which compared favourably to the protection induced by ischaemic preconditioning.([611;615])

Further studies are underway to determine whether shortening the duration of the 'ischaemic post-conditioning' protocol to 10 second episodes of alternate ischaemia/reperfusion is protective in the Langendorff-perfused rat heart, and studies are required to elucidate the mechanism of protection in this setting. Shorter episodes of ischaemia/reperfusion could potentially protect the heart by decreasing the opening probability of the mPTP. Whether the requirement for shorter episodes of ischaemia/reperfusion can be explained by the modulation of survival kinases such as MEK1/2-Erk1/2, as suggested by Downey's laboratory, is unclear.([615]) Other potential mechanisms by which 'ischaemic post-conditioning', could protect the heart against lethal reperfusion injury include the production of nitric oxide,([615]) attenuation of ROS production and reduced mitochondrial calcium load,([616;617]) all factors which could potentially protect the myocardium by inhibiting mPTP opening.

4.5 Conclusion

In this section of the thesis, we have demonstrated that the opening of the mPTP during the first 15 minutes of reperfusion, following a period of lethal ischaemia, is a critical determinant of cell death, as evidenced by the large reduction in infarct size associated with inhibiting mPTP opening using the known mPTP inhibitors, CsA or SfA. Interestingly, we demonstrated that these drugs were only effective if given for the first 15 minutes of reperfusion when compared to their administration after the first 15 minutes of reperfusion had elapsed, suggesting that treatment has to be directed to the first few minutes of reperfusion, when mPTP opening has been demonstrated to occur. Given the magnitude of the protection associated with inhibiting mPTP opening at the time of reperfusion, in the next chapter we explored whether this powerful protective mechanism contributed to the protection associated with the phenomenon of myocardial preconditioning.

The data from this part of the thesis demonstrating that inhibiting mPTP opening at the time of reperfusion results in a reduction in infarct size has been published.([873;874])

Chapter Five

THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE AS A TARGET FOR PROTECTION IN MYOCARDIAL PRECONDITIONING

5.1 Introduction

In the previous chapter, it was demonstrated that the opening of the mPTP during the first few minutes of reperfusion is a critical determinant of cell death in the setting of ischaemia-reperfusion injury. Furthermore, it was shown that inhibiting mPTP opening at the time of reperfusion could protect the heart against lethal reperfusion injury as evidenced by a reduction in infarct size following an episode of sustained ischaemia. In this chapter, the role of the mPTP in the phenomenon of myocardial preconditioning was examined to determine whether mPTP opening at the time of reperfusion is modulated in the setting of preconditioning. Specifically, we wanted to examine whether the protection associated with myocardial preconditioning is mediated by the inhibition of mPTP opening.

Interestingly, many of the factors that act to induce mPTP opening at the time of reperfusion are modified in the setting of preconditioning. For example, mitochondrial Ca^{2+}, which is a major determinant of mPTP opening at the time of reperfusion, has been demonstrated to be reduced in the setting of preconditioning induced by either ischaemia([238;239]) or opening of the mitochondrial K_{ATP} channel. ([52;239;257;258;261;262]) In addition, IPC and mitochondrial K_{ATP} channel openers have been demonstrated to preserve mitochondrial function and render mitochondria more tolerant to mitochondrial Ca^{2+} loading.([263-265])

Another major determinant of mPTP opening at the time of reperfusion is the oxidative stress which is generated primarily from the reoxygenation of an ischaemia-mediated reduced electron transport chain.([79;276;277]) Interesting, preconditioning induced by ischaemia,([278]) hypoxia,([79]) adenosine,([77;79]) or the K_{ATP} channel openers (diazoxide and nicorandil)([78]) have all been demonstrated to reduce the production of oxidative stress at the time of reperfusion.

Preserving mitochondrial energy production during ischaemia-reperfusion is critical for the survival and recovery of myocytes following an episode of ischaemia-reperfusion injury, and IPC has been demonstrated to preserve mitochondrial energy production during ischaemia-reperfusion injury.([228-231;231-234]) In addition, the opening of the mitochondrial K_{ATP} channel has been demonstrated to increase ATP synthesis,([234]), preserve mitochondrial energy production,([267]) decrease ATP hydrolysis,([159]) and improve energy transfer at reperfusion.([159])

Therefore protecting the heart against lethal reperfusion injury can be mediated by attenuating mitochondrial Ca^{2+} loading, reducing oxidative stress at the time of reperfusion, and

preserving mitochondrial energy production, during ischaemia-reperfusion, such that mPTP opening is suppressed at the time of reperfusion. In this section of the study we wanted to investigate whether myocardial preconditioning protects the hearts by inhibiting the mPTP opening that occurs in the first few minutes of reperfusion. At the time these experiments were performed the only published study directly related to this research area was a study by Ashraf's group([251]) in which they demonstrated using neonatal rat myocytes that the mPTP opener, atractyloside could reverse the protection associated with Ca^{2+}-preconditioning. However, in that study the effect of Ca^{2+}-preconditioning on mPTP opening was not examined. Therefore, the aim of this part of the study was to determine whether myocardial preconditioning protects the heart by inhibiting the mPTP opening that occurs at the time of reperfusion.

5.2 Hypothesis

Myocardial preconditioning protects by inhibiting the prolonged (high-conductance) mPTP opening at the time of reperfusion, following the lethal ischaemic insult.

The first part of the study (section 5.3) was to determine whether there existed any interplay between the mitochondrial K_{ATP} channel (a proposed mediator/effector of preconditioning) and the mPTP. Both are constituents of the inner mitochondrial membrane and are therefore subjected to the same ionic and metabolic changes during ischaemia-reperfusion injury. Having demonstrated in chapter 4, that inhibiting mPTP opening at the time of reperfusion, using CsA, could protect the heart against lethal reperfusion injury, we wanted to next determine whether modulating the mitochondrial K_{ATP} channel could influence the protection associated with CsA.

In the next part of the study (section 5.4), we adopted an approach similar to that used by Ashraf's group,([251]) in that we administered the mPTP opener atractyloside, to induce the opening the mPTP during the first 15 minutes of reperfusion, to determine whether we could abrogate the protection associated with ischaemic and pharmacological classical preconditioning, assuming that myocardial preconditioning had resulted in the suppression of mPTP opening. Because atractyloside is an inhibitor of ANT, which would interfere with the mitochondrial exchange of adenine nucleotides at the time of reperfusion, we also investigated an alternative mPTP opener, phenylarsenine oxide. The same approach was used to investigate the role of the mPTP in the second window of protection induced by the adenosine A1-receptor agonist, CCPA (section 5.5). Of course, these initial experiments do not examine mPTP opening directly, and they only suggest the involvement. The next objective therefore was to examine the effect of preconditioning on mPTP opening directly, using two different models for assessing mPTP opening.

In section 5.6 we examined the effect of pharmacological preconditioning using diazoxide on the mPTP-opening induced by Ca^{2+}, in adult rat mitochondria. Following this set of experiments, we examined the effect of hypoxic and pharmacological preconditioning on the mPTP opening-induced by oxidative stress in adult rat myocytes (section 5.7). In section 5.8, we explore the role of mitochondrial K_{ATP} channel opening at the time of reperfusion, which has been demonstrated to protect the heart against lethal reperfusion injury, to determine whether protection is mediated by inhibition of mPTP opening.

In the final section of this chapter (section 5.9), we investigate how myocardial preconditioning might modulate mPTP opening by examining the effects of the preconditioning mimetic, diazoxide, on mitochondrial function.

5.3 Aim (1)

Determine whether there is any interplay between the mPTP and the mitochondrial K_{ATP} channel

5.3.1 Materials

Cyclosporin-A (Sigma Chemicals, Poole, Dorset) was dissolved in 50% ethanol and added to the Krebs-Henseleit buffer such that the final ethanol concentration was less than 0.005%. Glibenclamide (Sigma Chemicals, Poole, Dorset) and glimepiride (Sigma Chemicals, Poole, Dorset) were dissolved in dimethyl sulphoxide (DMSO, Sigma Chemicals, Poole, Dorset) and added to the Krebs-Henseleit buffer such that the final DMSO concentration was less than 0.01%. 5-Hydroxydecanoic acid (5-HD, Sigma Chemicals, Poole, Dorset) and HMR 1098 (gift courtesy of Dr Jim Downey) were dissolved in distilled H_2O. All other reagents were of standard analytical grade.

5.3.2 Isolated Langendorff-Perfused Rat Heart Model

Hearts were excised from male Sprague-Dawley rats and mounted on a Langendorff-perfusion apparatus and subjected to 35 minutes regional ischaemia followed by 120 minutes of reperfusion (see section 3.2.1). At the end of the reperfusion period, the infarct-risk volume ratio was determined using tetrazolium (TTC) staining (see section 3.2.2).

5.3.3 Experimental Protocols for Infarct Studies

The experiment protocols for the infarct studies are presented in figure 5.1. The hearts were randomly assigned to one of the following treatment groups:

(1) ***Control hearts*** were perfused with 0.01% DMSO (n=3) or Krebs-Henseleit buffer alone (n=6) throughout stabilisation and ischaemia up until the point of reperfusion;

(2) ***CsA-treatment***: hearts (n=6) were perfused with cyclosporin-A (0.2 μmol/L) for the first 15 minutes of reperfusion. This concentration of CsA has been demonstrated to inhibit mPTP opening in the isolated perfused rat heart.([780])

(3) ***5-HD + CsA-treatment***: hearts (n=6) were perfused with 5-HD (100 μmol/l) throughout stabilisation and ischaemia up until the point of reperfusion and cyclosporin-A (0.2 μmol/L) was

given for the first 15 minutes of reperfusion. This concentration of 5-HD has been used to close the mitochondrial K_{ATP} channel in the isolated perfused rat heart;([104])

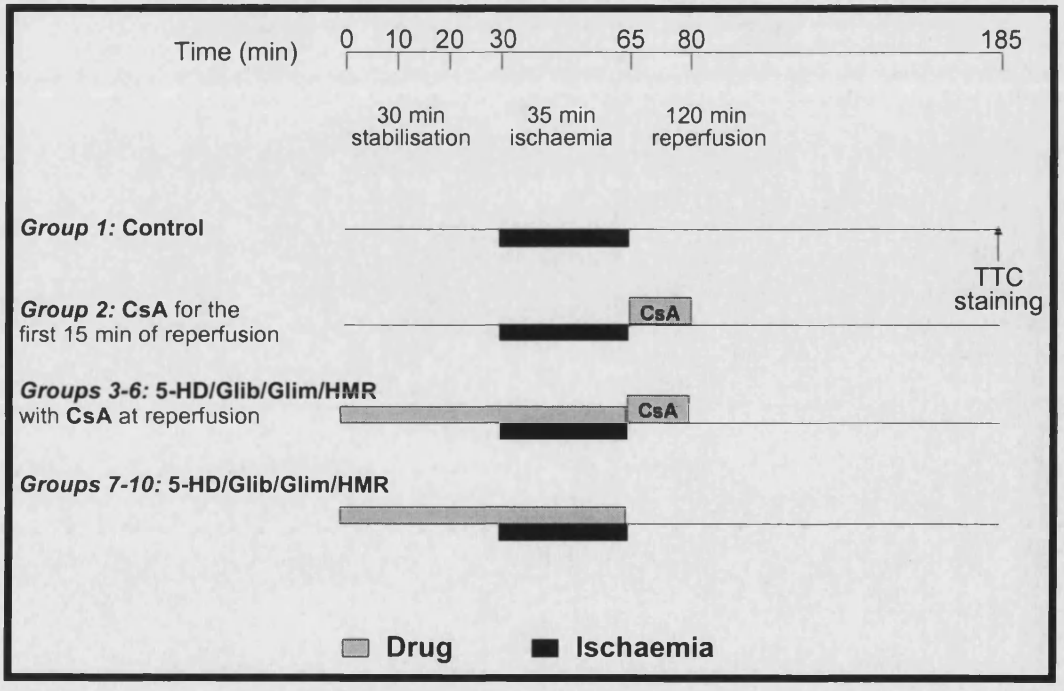
(4) ***Glibenclamide + CsA-treatment***: hearts (n=6) were perfused with glibenclamide (10 µmol/l) throughout stabilisation and ischaemia up until the point of reperfusion and cyclosporin-A (0.2 µmol/L) was given for the first 15 minutes of reperfusion. This concentration of glibenclamide has been used to close the K_{ATP} channel in the isolated perfused rat heart;([875])

(5) ***Glimepiride + CsA-treatment***: hearts (n=6) were perfused with glimepiride (10 µmol/l) throughout stabilisation and ischaemia up until the point of reperfusion and cyclosporin-A (0.2 µmol/L) was given for the first 15 minutes of reperfusion. This concentration of glimepiride has been used to close the K_{ATP} channel in the isolated perfused rat heart;([875])

(6) ***HMR 1098 + CsA-treatment***: hearts (n=6) were perfused with HMR 1098 (10 µmol/l) throughout stabilisation and ischaemia up until the point of reperfusion and cyclosporin-A (0.2 µmol/L) was given for the first 15 minutes of reperfusion. This concentration of HMR 1098 has been used to close the sarcolemmal K_{ATP} channel in the isolated perfused rat heart;([876])

(7-10) ***5-HD/Glibenclamide/Glimepiride/HMR 1098-treatment***: hearts were perfused with either 5-HD (100 µmol/l, n=4), glibenclamide (10 µmol/l, n=5), glimepiride (10 µmol/l, n=4) or HMR 1098 (10 µmol/l, n=4) throughout stabilisation and ischaemia up until the point of reperfusion;

Figure 5.1: Experimental Protocols Investigating the Interplay Between the mPTP and the Mitochondrial K_{ATP} Channel. 5-HD-5-hydroxydecanoic acid, Glib-glibenclamide, Glim-Glimepiride, HMR-HMR 1098, TTC-tetrazolium.



5.3.4 Results

5.3.4.1 Exclusions

We used 56 male Sprague-Dawley rat hearts for the infarct size experiments of which 3 were excluded owing to poor function during stabilisation (see section 3.2.3 for exclusion criteria).

5.3.4.2 Animal and Haemodynamic Data

Animal and heart weights and risk zone volumes were similar in the experimental groups (see table 5.1). Baseline data relating to cardiac function (assessed by the rate-pressure-product, RPP) and coronary flow before the lethal ischaemic period, were similar in the experimental groups (see table 5.2, 5.3). During regional ischaemia, coronary flow and RPP decreased to a similar extent in both groups. An increase in cardiac function and coronary flow upon reperfusion was indicative of successful re-flow.

Table 5.1 Characteristics of Animals in Treatment Groups

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
1. Control	6	359±19	1.90±0.06	0.411±0.017
2. CsA	6	387±20	1.98±0.07	0.555±0.037
3. 5-HD + CsA	6	358±2	1.82±0.06	0.533±0.059
4. Glib + CsA	6	331±10	1.67±0.09	0.462±0.048
5. Glim + CsA	6	338±12	1.77±0.22	0.490±0.063
6. HMR +CsA	6	333±5	1.84±0.10	0.481±0.034
7. 5-HD	4	368±6	1.89±0.22	0.517±0.063
8. Glib	5	349±9	1.72±0.09	0.508±0.023
9. Glim	4	362±25	2.01±0.21	0.513±0.019
10. HMR	4	328±6	1.72±0.11	0.439±0.053

Values are mean±SEM.

Table 5.2 Rate Pressure Product (x 10^3mmHg/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	29.8±2.0	27.4±2.3	13.3±1.7	13.8±2.2	12.8±2.1	10.1±1.3
2. CsA	36.4±1.0	32.1±2.1	12.5±2.0	18.9±1.4	12.1±0.9	10.8±1.6
3. 5-HD + CsA	37.4±1.7	32.8±3.2	9.1±1.0	14.4±0.9	16.2±2.0	13.0±1.7
4. Glib + CsA	25.6±0.9	25.5±1.8	9.2±0.8	11.9±2.0	13.9±1.3	9.7±0.4
5. Glim + CsA	19.3±0.9*	27.0±1.8	8.3±0.3	13.6±1.8	12.7±1.3	12.3±0.8
6. HMR +CsA	28.4±2.7	31.6±2.5	9.4±2.5	13.6±3.7	12.7±2.9	15.7±0.6
7. 5-HD	28.8±0.5	27.8±4.3	10.9±1.2	10.4±1.8	13.5±2.0	9.3±0.6
8. Glib	25.0±1.3	21.3±1.8	9.0±1.0	13.1±1.4	12.7±2.1	9.9±0.5
9. Glim	22.9±3.1	21.9±6.8	14.0±0.8	12.3±0.3	16.0±3.5	14.3±2.6
10. HMR	32.6±4.0	30.6±4.4	11.2±1.3	12.4±2.2	11.3±0.2	10.1±1.1

Values are mean±SEM. *P<0.05 compared with control.

Table 5.3 Coronary Flow Rate (ml/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	14.6±1.1	12.6±1.2	7.2±0.9	6.0±0.8	8.8±1.4	5.2±0.7
2. CsA	15.8±1.4	13.6±0.7	7.2±0.9	6.0±0.5	7.0±1.0	5.0±0.6
3. 5-HD + CsA	16.0±0.6	14.7±0.9	8.0±0.6	6.7±0.3	8.0±0.8	6.3±0.3
4. Glib + CsA	11.8±0.7	11.2±0.4	6.2±0.7	5.6±0.5	8.0±0.6	6.0±1.0
5. Glim + CsA	15.2±1.0	14.6±1.2	6.4±0.5	5.8±0.6	9.5±1.3	6.3±0.9
6. HMR +CsA	15.5±0.6	15.0±0.7	10.8±2.8	8.5±0.3	9.5±0.3	5.8±0.5
7. 5-HD	15.3±0.8	14.0±1.4	8.3±0.3	5.3±0.3	7.7±0.9	5.0±0.6
8. Glib	12.5±1.3	12.5±1.2	6.8±0.5	6.0±0.7	8.3±1.7	5.0±0.6
9. Glim	15.3±1.1	13.0±1.5	6.8±1.0	6.5±1.2	9.3±1.6	6.0±1.0
10. HMR	15.3±2.7	14.7±2.4	5.7±0.9	4.7±0.7	8.0±1.5	5.7±0.3

Values are mean±SEM.

5.3.4.3 Infarct Size Data

Infarct size is presented as the percentage of tetrazolium-negative tissue in the ischaemic risk zone. The protection induced by inhibiting the prolonged mPTP opening that occurs at reperfusion, using CsA, was abolished in the presence of the mitochondrial selective K_{ATP} channel blocker, 5-HD (24.6±1.9% with CsA vs 47.1±3.5% with CsA and 5-HD; P<0.001; figure 5.2), the non-selective K_{ATP} channel blocker glibenclamide (24.6±1.9% with CsA vs 52.6±1.8% with CsA and glibenclamide; P<0.001; figure 5.2), the K_{ATP} channel blocker glimepiride (24.6±1.9% with CsA vs 41.7±3.0% with CsA and glimepiride; P<0.001; figure 5.2). However, the protection induced by CsA was maintained in the presence of the specific sarcolemmal K_{ATP} channel blocker, HMR 1098 (24.6±1.9% with CsA vs 29.4±0.5% with CsA and HMR 1098; P=NS; figure 5.2). The K_{ATP} channel blockers alone did not influence infarct size (49.3±4.1% with 5-HD, 47.1±4.2% with glibenclamide, 41.7±3.0% with glimepiride, 47.9±3.1% with HMR 1098 vs 44.8±3.2% in control; P=NS; figure 5.3).

Figure 5.2: Closing the K_{ATP} Channel Abrogates CsA-Induced Cardio Protection. Pharmacologically closing the K_{ATP} channel using 5-hydroxydecanoic acid (5-HD), glibenclamide (Glib) or glimepiride (Glim) during stabilisation and ischaemia abrogates the protection mediated by cyclosporin-A (CsA)-induced inhibition of mPTP opening at reperfusion. Pharmacologically closing the sarcolemmal K_{ATP} channel using HMR 1098 (HMR) had no effect on CsA-induced protection.*P<0.001 compared with control.

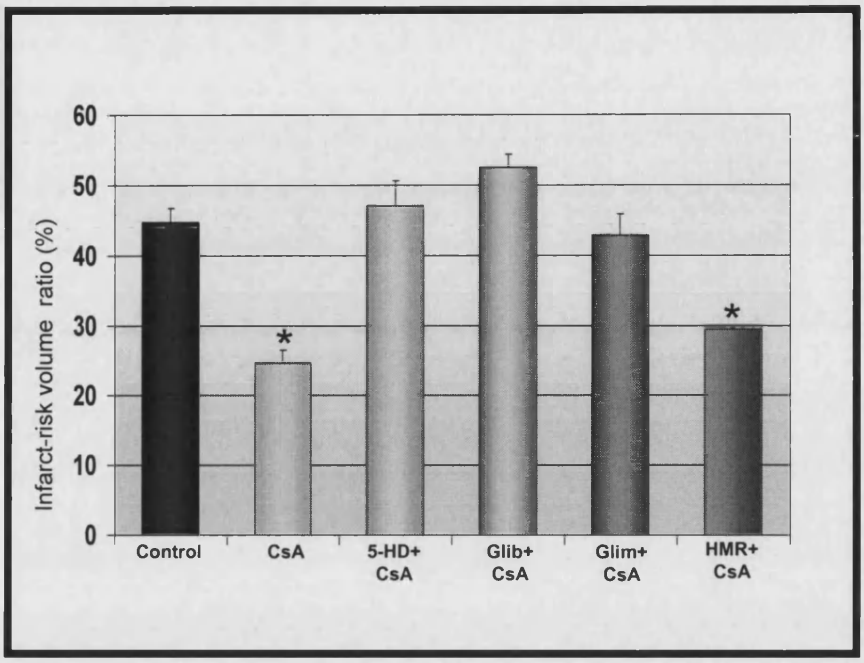
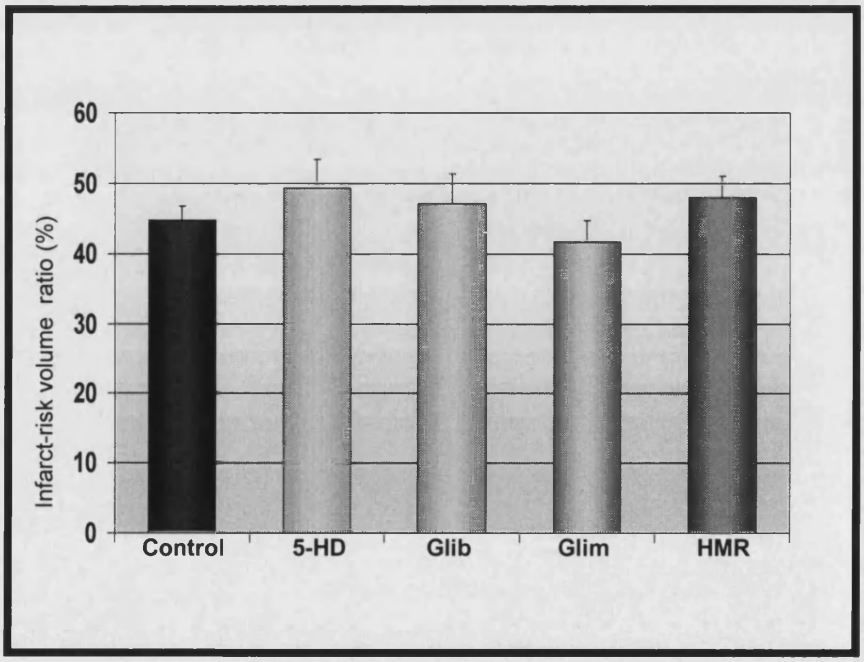


Figure 5.3: Closing the K_{ATP} Channel Does not Influence Infarct Size. Pharmacologically closing the mitochondrial K_{ATP} channel using 5-hydroxydecanoic acid (5-HD), glibenclamide (Glib) or glimepiride (Glim) or closing the sarcolemmal K_{ATP} channel using HMR 1098 (HMR) during stabilisation and ischaemia has no effect on infarct size. *P<0.001 compared with control.



5.3.5 Discussion

The results from this section of the study, suggest that there exists an interplay between the mitochondrial K_{ATP} channel and the mPTP such that the closing of the former during stabilisation and the index ischaemic period appeared to antagonise CsA-induced protection at the time of reperfusion. We demonstrated that the presence of glibenclamide or 5-HD (presumed mitochondrial K_{ATP} channel blockers) during the pre-ischaemic and ischaemic period abrogated the CsA-mediated reduction in infarct size. However, the sarcolemmal K_{ATP} channel blocker did not influence the CsA-mediated reduction in infarct size, suggesting that there is no apparent interplay between the sarcolemmal K_{ATP} channel and the mPTP.

However, the results with glimepiride are surprising. According to a recent study by Yellon's group,([875]) this K_{ATP} channel blocker does not target the mitochondria, as it did not block IPC or diazoxide-induced cardio-protection. Furthermore, glimepiride was not demonstrated to antagonise mitochondrial K_{ATP} channel opening as evidenced by the fact that it did not block the GTP-induced mitochondrial membrane depolarisation in isolated mitochondria.([875]) The finding that glimepiride does not abolish preconditioning has been demonstrated in rabbit hearts,([877]) preconditioning at the time of PTCA,([878]) and in diabetic rats.([879]) Therefore, why glimepiride should abrogate CsA-induced protection is unknown if the mitochondrial K_{ATP} channel is supposed to be the mediator.

The mechanism by which these K_{ATP} channel blockers abrogate CsA-mediated inhibition of mPTP opening at the time of reperfusion is unclear. We can speculate on several possibilities: (1) It may represent a drug interaction between the K_{ATP} channel blockers and CsA and the abrogation of CsA-induced cardio-protection or else a non-specific effect of the K_{ATP} channel blockers on mitochondrial function.([107;158]) Either way the observed effect may be independent of any effect on the K_{ATP} channel. This may explain the discrepancy with glimepiride also blocking CsA-induced protection, when it is believed to not influence the mitochondrial K_{ATP} channel; (2) The capacity of CsA to induce inhibition of mPTP opening depends on its ability to bind to cyclophilin D and prevents its interaction with the ANT.([666]) Therefore, factors which act to facilitate the binding of cyclophilin D to ANT, may overcome the inhibitory effect of CsA on mPTP opening. In this regard, Halestrap's group have demonstrated that the binding of cyclophilin D to ANT was facilitated by chaotropic agents (substances, usually ions [e.g., SCN-, ClO4-, guanidinium], which disrupt the structure of water and thereby

promote the solubility of non-polar substances in polar solvents [e.g., water], and promote the unfolding of proteins) and mitochondrial swelling.([671]) Intriguingly, the K_{ATP} channel blocker, 5-HD, has been demonstrated to induce mitochondrial matrix swelling,([149]) and so potentially it could enhance the binding of cyclophilin D to ANT, thereby overcoming the inhibitory effect of CsA on mPTP opening. Glibenclamide has been demonstrated to induce K^+ influx into mitochondria,([819]) which would be expected to increase matrix volume.

This part of the study suggested that there may be an interaction between the mitochondrial K_{ATP} channel (which has been implicated in preconditioning) and the mPTP (which has been implicated in ischaemia-reperfusion injury). Therefore, our next objective was to determine whether mPTP opening in the setting of ischaemia-reperfusion injury can be modulated by preconditioning.

5.4 Aim (2)

Determine whether pharmacologically opening the mPTP at the time of reperfusion abrogates classical preconditioning-induced protection

In this section we examined the effect of pharmacologically opening the mPTP for the first 15 minutes of reperfusion, using either atractyloside or phenylarsenine oxide, on the protection induced by both ischaemic and pharmacological preconditioning, based on the assumption that preconditioning protects by inhibiting mPTP opening at the time of reperfusion. Atractyloside is an ANT inhibitor which has been demonstrated to induce mPTP opening. Ashraf's group demonstrated that at 20 µmol/l, this drug reversed the protection associated with calcium preconditioning.([251]) Phenylarsenine oxide (PAO) induces mPTP opening by cross-linking critical thiol groups on the ANT,([655;658]) a component of the mPTP. This drug has been used in isolated myocytes to induce mPTP opening as detected by the movement of the fluorescent dye, calcein out of the mitochondria.([857])

In this part of the study we also wanted to confirm that the protection associated with IPC and diazoxide could be abolished by the mitochondrial K_{ATP} channel blocker, 5-hydroxydecanoate.

5.4.1 Materials

Diazoxide (Sigma Chemicals, Poole, Dorset) was dissolved in dimethyl sulfoxide (DMSO) and added to the Krebs-Henseleit buffer such that the final DMSO concentration was less than 0.02%. 2 chloro-N^6-cyclopentyl-adenosine (CCPA, Sigma Chemicals, Poole, Dorset), atractyloside (Atr, Sigma Chemicals, Poole, Dorset), and 5-hydroxydecanoic acid (5-HD, Sigma Chemicals, Poole, Dorset) were dissolved in distilled water and added to the Krebs-Henseleit buffer. All other reagents were of standard analytical grade.

5.4.2 Isolated Langendorff-Perfused Rat Heart Model

Hearts were excised from male Sprague-Dawley rats and mounted on a Langendorff-perfusion apparatus and subjected to 35 minutes regional ischaemia followed by 120 minutes of reperfusion (see section 3.2.1). At the end of the reperfusion period, the infarct-risk volume ratio was determined using tetrazolium (TTC) staining (see section 3.2.2).

5.4.3 Experimental Protocols for Infarct Studies

The experiment protocols for the infarct studies are presented in figure 5.4. The hearts were randomly assigned to one of the following treatment groups:

(1) ***Control hearts*** were perfused with 0.02% DMSO (n=3) or Krebs-Henseleit buffer alone (n=6) for 10 minutes prior to lethal ischaemia;

(2) ***Ischaemic preconditioning (IPC)-treated hearts*** (n=6) were treated with 2 periods of 5 minutes each of global ischaemia with a 10-minute intervening reperfusion before the lethal ischemia;

(3) ***Diazoxide-treatment:*** hearts (n=6) were perfused with diazoxide (30 μmol/l) for 10 minutes followed by 10 minutes washout with Krebs-Henseleit buffer prior to lethal ischemia. This concentration of diazoxide has been previously shown to pharmacologically precondition the isolated perfused rat heart;([104])

(4) ***CCPA-treatment:*** hearts (n=7) were perfused with CCPA (200 nmol/l) for 10 minutes (during which time the hearts were paced at 300 beats per minute due to CCPA-induced bradycardia) followed by 10 minutes washout with Krebs-Henseleit buffer prior to lethal ischemia. This concentration of CCPA has been shown to precondition the isolated perfused rat heart;([880])

(5) ***IPC + atractyloside-treatment:*** hearts (n=6) underwent IPC (as in group 2) and were then perfused with atractyloside (20 μmol/l, a known opener of the mPTP)([8-10]) for the first 15 minutes of reperfusion. This concentration of atractyloside has been previously demonstrated to reverse the effects of calcium-preconditioning in adult rat myocytes;([251])

(6) ***Diazoxide + atractyloside-treatment:*** hearts (n=6) were pre-treated with diazoxide (as in group 3) and were then perfused with atractyloside for the first 15 minutes of reperfusion;

(7) ***CCPA + atractyloside-treatment:*** hearts (n=6) were pre-treated with CCPA (as in group 4) and were then perfused with atractyloside for the first 15 minutes of reperfusion;

(8) ***Atractyloside-treatment:*** hearts (n=6) were perfused with atractyloside for the first 15 minutes of reperfusion;

(9) ***IPC + phenylarsenine oxide-treatment:*** Hearts (n=6) underwent IPC (as in group 2) and were perfused with phenylarsenine oxide (PAO, 2 μmol/l, n=3), for the first 15 minutes of reperfusion. PAO is another opener of the mPTP and was used here to induce prolonged opening of the mPTP for the first 15 minutes of reperfusion. A PAO concentration of 20 μM had

been shown to induce mPTP opening in myocytes.([857]) At this concentration the hearts in our study went into immediate irreversible hypercontracture. Hence, we used the lower concentration of 2 μmol/l.

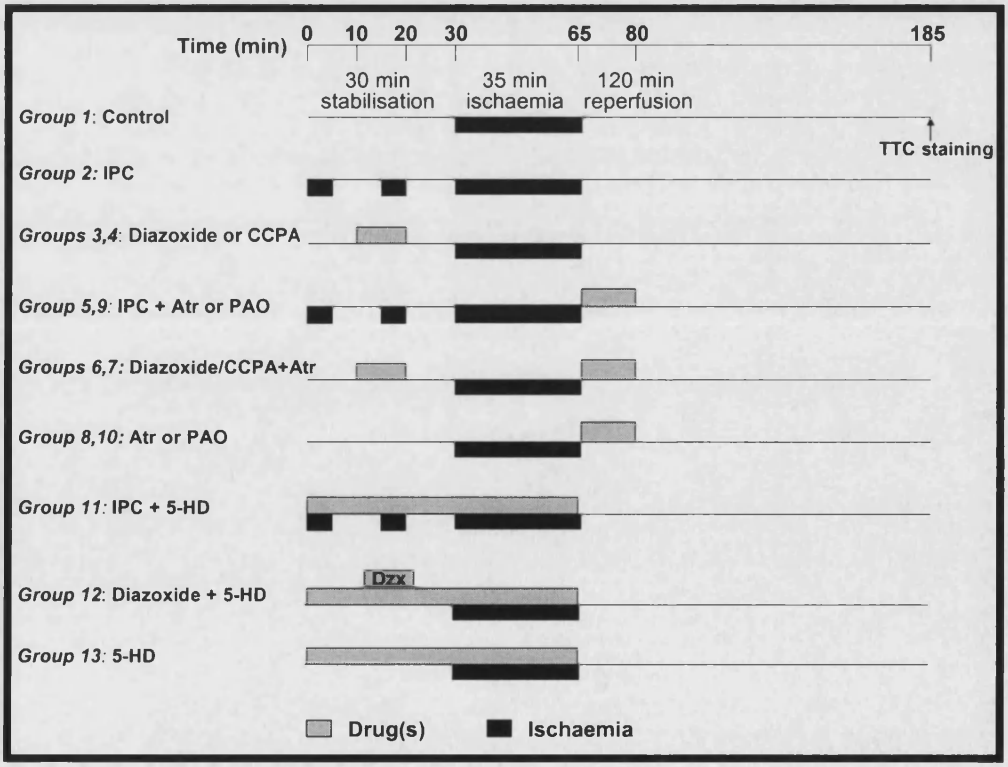
(10) ***Phenylarsenine oxide-treatment:*** Hearts (n=3) were perfused with phenylarsenine oxide (PAO, 2 μmol/l, n=3), for the first 15 minutes of reperfusion.

(11) ***IPC + 5-HD-treatment:*** hearts (n=6) underwent IPC (as in group 2) in the presence of 5-HD (100 μmol/L) prior to and during regional ischaemia. This concentration of 5-HD has been shown to block preconditioning in the isolated perfused rat heart;([104])

(12) ***Diazoxide + 5-HD-treatment:*** hearts (n=6) were preconditioned with diazoxide (as in group 3) in the presence of 5-HD (100 mol/L) which was perfused throughout ischaemia.

(13) ***5-HD-treatment:*** hearts (n=4) were perfused with 100 μmol/L 5-HD prior to and during regional ischaemia.

Figure 5.4: ***Experimental Protocols Investigating the Effect of Opening the mPTP at the Time of Reperfusion in Preconditioned Hearts***. 5-HD-5-hydroxydecanoic acid, Atr-Atractyloside, Dzx-Diazoxide, TTC-tetrazolium, CCPA-2 chloro-N^6-cyclopentyl-adenosine. PAO- phenylarsenine oxide.



5.4.4 Results

5.4.4.1 Exclusions

We used 82 male Sprague-Dawley rat hearts for the infarct size experiments of which 8 were excluded owing to poor function during stabilisation (see section 3.2.3 for exclusion criteria), including 3 which were excluded due to phenylarsenine oxide-induced irreversible hypercontracture at the concentration of 20 µmol/l.

5.4.4.2 Animal and Haemodynamic Data

Animal and heart weights and risk zone volumes were similar in the experimental groups (see table 5.4). Baseline data relating to cardiac function (assessed by the rate-pressure-product, RPP) and coronary flow before the lethal ischaemic period, were similar in the experimental groups (see table 5.5, 5.6). During regional ischaemia, coronary flow and RPP decreased to a similar extent in both groups. An increase in cardiac function and coronary flow upon reperfusion was indicative of successful re-flow.

Table 5.4 Characteristics of Animals in Treatment Groups

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
1. Control	6	419±2	1.93±0.08	0.605±0.030
2. IPC	6	405±10	2.06±0.05	0.504±0.051
3. Dzx	6	395±7.1	2.11±0.01	0.565±0.046
4. CCPA	7	374±8.9	2.20±0.21	0.562±0.030
5. IPC + Atr	6	358±37	2.08±0.15	0.578±0.030
6. Dzx + Atr	6	399±18	2.20±0.21	0.559±0.046
7. CCPA + Atr	6	368±13	1.94±0.13	0.530±0.029
8. Atr	6	363±11	2.00±0.13	0.528±0.027
9. IPC + PAO	6	398±9	1.62±0.06	0.474±0.022
10. PAO	3	372±2	2.12±0.03	0.513±0.078
11. IPC + 5-HD	6	343±10	1.77±0.07	0.513±0.078
12. Dzx + 5-HD	6	338±10	1.72±0.05	0.508±0.029
13. 5-HD	4	368±6	1.89±0.22	0.517±0.063

Values are mean±SEM. *P<0.001 compared with control.

Table 5.5 Rate Pressure Product (x 10^3mmHg/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	26.5±1.8	25.3±2.8	7.2±1.5	14.0±2.6	17.2±2.8	9.9±1.5
2. IPC	28.1±4.1	26.4±1.2	8.1±1.3	15.3±0.8	9.3±2.3	9.0±1.5
3. Dzx	26.3±1.7	24.7±1.4	5.1±0.6	12.1±1.8	13.7±2.3	9.4±1.8
4. CCPA	26.3±3.3	24.9±4.6	7.1±0.8	15.5±2.0	18.6±3.3	6.4±1.2
5. IPC + Atr	25.6±2.2	25.9±0.8	7.8±1.2	8.9±0.8	10.2±0.4	6.5±0.6
6. Dzx + Atr	23.3±1.6	23.3±1.4	6.1±0.8	8.9±1.6	9.2±1.4	3.9±1.0
7. CCPA + Atr	26.4±2.4	26.8±2.6	7.8±1.5	10.0±1.5	10.6±0.7	6.4±1.2
8. Atr	23.0±1.2	27.0±2.5	8.4±1.4	13.0±2.2	9.6±3.1	6.5±1.2
9. IPC + PAO	25.5±5.4	26.0±3.0	7.1±2.2	16.4±0.4	19.3±0.4	8.1±1.1
10. PAO	21.6±2.8	28.1±2.0	8.7±1.7	13.4±0.6	11.3±4.3	4.4±0.4
11. IPC + 5-HD	27.9±3.2	29.8±8.8	8.8±1.9	14.9±3.2	19.7±1.1	13.4±1.7
12. Dzx + 5-HD	33.8±1.9*	31.8±1.2	5.8±0.8	13.1±2.4	21.4±3.2	18.0±2.1
13. 5-HD	37.4±1.7*	32.8±3.2*	9.1±1.0	14.4±0.9	16.2±2.0	13.0±1.7

Table 5.6 Coronary Flow Rate (ml/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	18.2±0.5	17.6±0.5	7.4±0.2	7.4±0.2	10.0±1.0	6.6±0.7
2. IPC	17.3±2.4	16.0±2.1	7.3±0.7	7.3±0.7	11.5±0.5	8.5±1.5
3. Dzx	17.0±1.1	15.8±1.0	6.2±0.4	5.8±0.2	9.2±0.9	4.8±0.5
4. CCPA	15.4±1.7	16.3±1.9	5.5±0.3	5.5±0.3	9.3±1.0	5.3±0.6
5. IPC + Atr	15.2±0.7	14.8±0.6	6.6±0.7	5.6±0.5	8.0±0.6	5.0±0.3
6. Dzx + Atr	15.5±1.2	14.3±1.1	5.3±0.6	5.3±0.8	9.0±1.8	5.8±1.4
7. CCPA + Atr	14.5±0.9	14.0±1.2	5.0±0.6	4.8±0.5	7.0±0.4	4.3±0.3
8. Atr	15.8±1.3	15.0±1.7	5.8±0.3	5.3±0.3	8.5±0.6	4.3±0.3
9. IPC + PAO	19.3±1.7	19.7±0.3	8.7±0.3	8.0±0.3	16.0±1.1	8.5±1.5
10. PAO	17.0±3.0	17.0±3.0	9.5±1.5	10.0±1.0	14.0±2.0	8.5±1.5
11. IPC + 5-HD	18.7±1.0	19.5±0.5	8.5±0.2	7.8±0.6	12.2±0.6	8.6±0.8
12. Dzx + 5-HD	20.3±0.3	20.0±0.8	6.5±0.5	6.8±0.5	12.8±1.1	8.0±1.4
13. 5-HD	15.3±0.8	14.0±1.4	8.3±0.3	5.3±0.3	7.7±0.9	5.0±0.6

Values are mean±SEM. *P<0.05 compared with control.

5.4.4.3 Infarct Size Data

Infarct size is represented as the percentage of tetrazolium-negative tissue in the ischaemic risk zone. IPC significantly reduced infarct size compared with control hearts (19.9±2.6% with IPC vs 44.7±2.0% in control, P <0.0001; figure 5.5), as did pharmacological preconditioning using diazoxide (18.0±1.7% with diazoxide vs 44.7±2.0% in control, P<0.0001; figure 5.5) or the adenosine A1-receptor agonist, CCPA (20.4±3.3% with CCPA vs 44.7±2.0% in control, P<0.0001; figure 5.5). However, when atractyloside (the mPTP opener) was administered after 35 minutes ischaemia and immediately at reperfusion for 15 minutes, it abolished the protective effects of IPC (47.7±1.8% with IPC + Atr vs 19.9±2.6% with IPC, P<0.0001; figure 5.5), diazoxide (42.3±3.2% with diazoxide + Atr vs 18.0±1.7% with diazoxide, P<0.0001; figure 5.5) and CCPA (51.2±1.6% with CCPA + Atr vs 20.4±3.3% with CCPA, P<0.0001; figure 5.5). Atractyloside given alone for the first 15 minutes of reperfusion did not influence infarct size (43.9±1.5% vs 44.7±2.0% with control; P=NS; figure 5.5).

Opening the mPTP for the first 15 minutes of reperfusion using phenylarsenine oxide (PAO) also abrogated the protection induced by IPC (45.9±4.6% in IPC+PAO vs 19.9±2.6% in IPC; N=3; P<0.001; figure 5.5). PAO given alone for the first 15 minutes of reperfusion did not influence infarct size (48.3±3.2% vs 44.7±2.0% with control; P=NS; figure 5.5).

The presence of the mitochondrial K_{ATP} channel blocker, 5-HD prior to and during the lethal ischaemic period abolished the cardio-protective effects of both IPC (45.8±3.7% with IPC + 5-HD vs 19.9±2.6% with IPC, P<0.0001; figure 5.6) and diazoxide (46.6±2.5% with diazoxide + 5-HD vs 18.0±1.7% with diazoxide, P<0.0001; figure 5.6). 5-HD given alone prior to and during the lethal ischaemic period did not influence infarct size (49.3±4.1% with 5-HD vs 44.7±2.0% with control; P=NS; figure 5.6).

Figure 5.5: ***Pharmacologically Opening the mPTP at Reperfusion Abrogates Preconditioning-Induced Protection.*** Pharmacologically opening the mPTP using atractyloside (Atr) abrogated the protection associated with ischaemic preconditioning (IPC), diazoxide (Dzx), and 2 chloro-N^6-cyclopentyl-adenosine (CCPA). Pharmacologically opening the mPTP using phenylarsenine oxide (PAO) also abrogated the protection associated with ischaemic preconditioning (IPC) *P<0.001 compared with control.

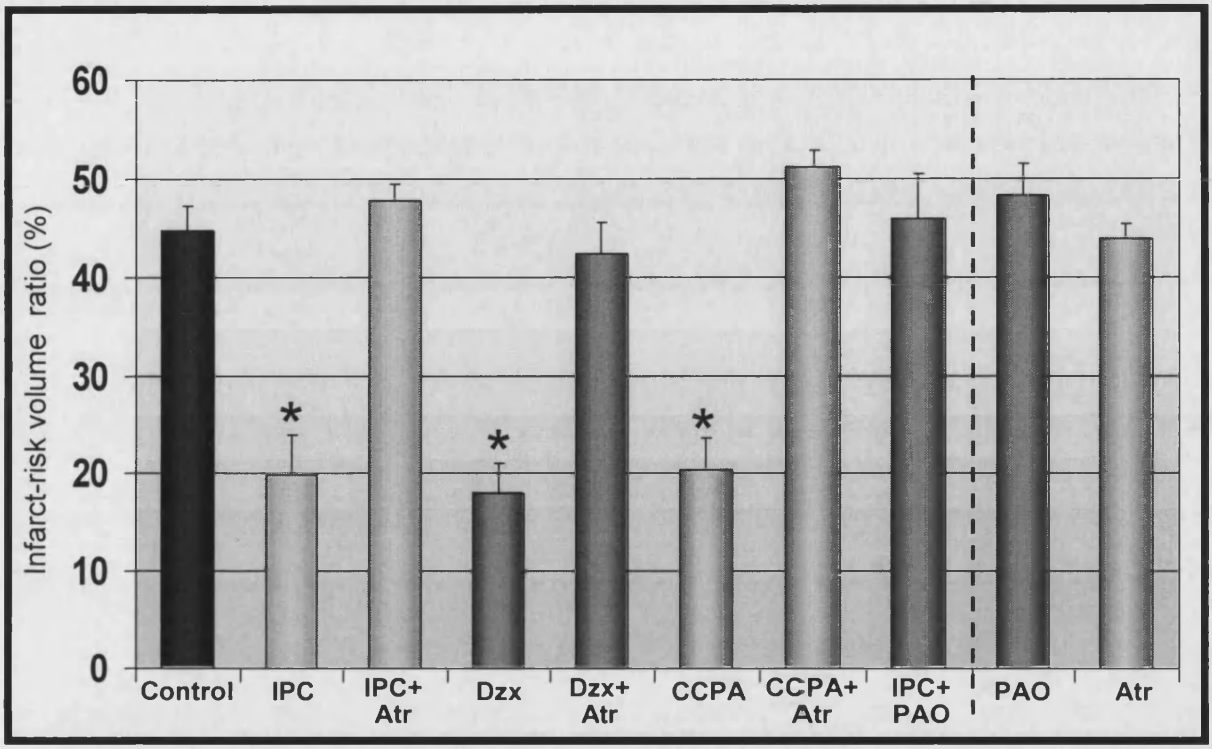
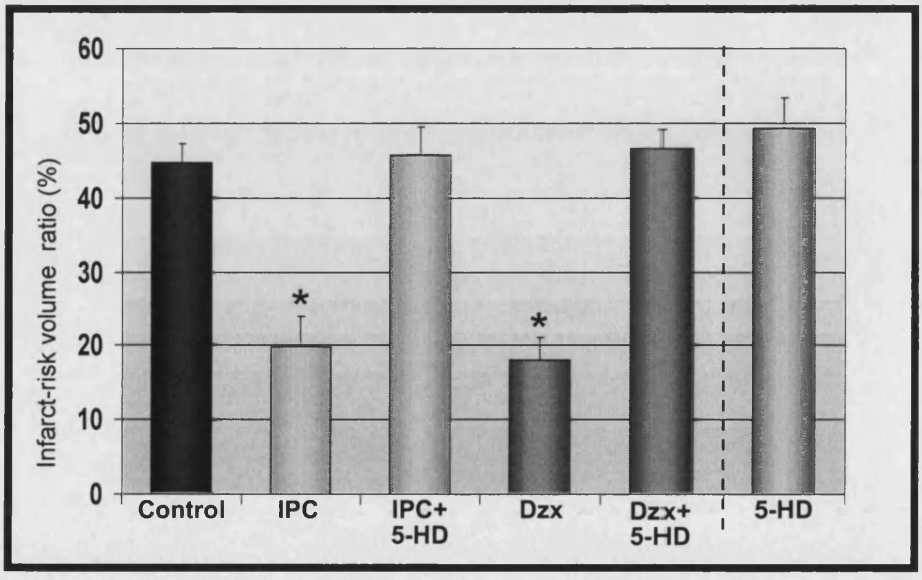


Figure 5.6: ***Pharmacologically Closing the Mitochondrial K_{ATP} Channel Abrogates Preconditioning Induced Protection:*** Closing the mitochondrial K_{ATP} Channel using 5-hydroxydecanoic acid (5-HD) abolishes both ischaemic preconditioning (IPC) and diazoxide (Dzx)-induced protection.*P<0.001.



5.4.5 Discussion

In this part of the study, we demonstrated that opening the mPTP during the first 15 minutes of reperfusion, following a period of lethal ischaemia, abrogated the protection induced by ischaemic preconditioning and pharmacological preconditioning using either diazoxide or the adenosine A1 receptor agonist, CCPA. It was important to demonstrate that two different mPTP openers were able to abolish the protection induced by IPC, especially since atractyloside, an inhibitor of ANT, would therefore be expected to inhibit the mitochondrial transfer of ATP to the cytosol during the first few minutes of reperfusion.

Interestingly, the opening the mPTP for the first 15 minutes of reperfusion increased the infarct size in hearts which had been preconditioned to a level comparable with control hearts, which would suggest that these drugs do not appear to non-specifically induce mPTP opening. In support of this, is the fact that the presence of either Atr or PAO during the first 15 minutes of reperfusion in control hearts did not influence infarct size. If these drugs were non-specifically inducing mPTP opening, one may have expected to have obtained an increase in infarct size compared to the control hearts. Interestingly, a study by Baines and colleagues also demonstrated that atractyloside did not increase the infarct size when compared to control hearts,([881]) as did a study by Rajesh and colleagues.([882]) In addition, the latter study found no increase in infarct size with isolated perfused rats heart given another mPTP opener Ionidamine.([882])

This may suggest that these mPTP openers have been targeting the mPTP's that had been inhibited by the process of preconditioning. Of course this is pure speculation and the abrogation of preconditioning-induced protection observed with the mPTP openers, Atr and PAO may solely be due to the fact that by opening the mPTP one causes necrotic cell death. However, we found that perfusing isolated rat hearts not subjected to ischaemia, with atractyloside did not produce any infarction, as measured by tetrazolium staining, suggesting the explanation may not be quite so simple. Interestingly, a study by Pastorino and colleagues([755]) using isolated mitochondria, observed that atractyloside only induced mPTP opening in the presence of the apoptotic protein, Bax (which translocates to mitochondria in response to an apoptotic stimulus such as ischaemia-reperfusion).

The finding that opening the mPTP using atractyloside abrogates IPC-induced protection has been confirmed in the isolated mouse heart by Baines and colleagues.([881])

However, a study by Rajesh and co-workers failed to block IPC-induced protection, using the open-chest rat model,([882]) yet they were able to block the effects of the SWOP (see section 5.5). The reason for the discrepancy between their results and our in unclear but may relate to a difference in the treatment protocols and the model used.

We used an isolated perfused rat heart and gave the mPTP openers, atractyloside or PAO at the time of reperfusion, as this is the time period when mPTP opening has been demonstrated to occur in the setting of ischaemia-reperfusion injury. In contrast, the study by Rajesh and colleagues used the open chest rat model and they gave the mPTP openers atractyloside and Ionidamine prior to the index ischaemic episode.([882])

In our set of experiments we also demonstrated that IPC and diazoxide induced protection could be inhibited by 5-hydroxydecanoic acid, the putative mitochondrial K_{ATP} channel blocker, supporting the findings of Schultz and colleagues.([883])

Having demonstrated that classical preconditioning may modulate the mPTP, our next objective was to examine whether the mPTP was modulated in the second window of protection.

5.5 Aim (3)

Determine whether pharmacologically opening the mPTP at the time of reperfusion abrogates the Second Window of Protection

In this part of the study, we investigated whether mPTP opening is modulated in the second window of protection (SWOP). SWOP was induced by giving the adenosine A1-receptor agonist CCPA, 24 hours before subjecting isolated perfused rats hearts to ischaemia-reperfusion. Again the drug atractyloside was given for the first 15 minutes of reperfusion to open the mPTP.

5.5.1 Materials

2 chloro-N^6-cyclopentyl-adenosine (CCPA, Sigma Chemicals, Poole, Dorset), was dissolved in normal saline. Atractyloside (Atr, Sigma Chemicals, Poole, Dorset) was dissolved in distilled water. All other reagents were of standard analytical grade.

5.5.2 Isolated Langendorff-Perfused Rat Heart Model

Hearts were excised from male Sprague-Dawley rats and mounted on a Langendorff-perfusion apparatus and subjected to 35 minutes regional ischaemia followed by 120 minutes of reperfusion (see section 3.2.1). At the end of the reperfusion period, the infarct-risk volume ratio was determined using tetrazolium (TTC) staining (see section 3.2.2).

5.5.3 Experimental Protocols for Infarct Studies

The experiment protocols for the infarct studies are presented in figure 5.7. The hearts were randomly assigned to one of the following treatment groups:

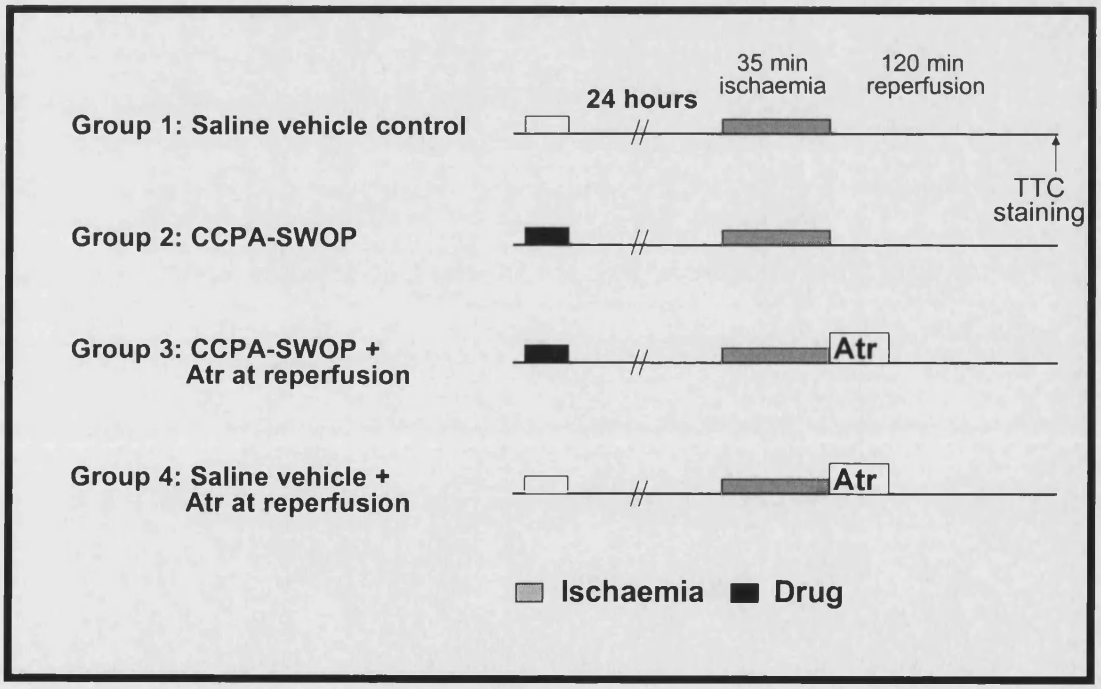
(1) ***Control hearts***: Rats (n=6) received a tail-vein injection of saline vehicle and 24 hours later the hearts were excised and Langendorff-perfused and subjected to 35 minutes lethal ischaemia and 120 minutes reperfusion;

(2) ***CCPA-Induced SWOP***: Rats received a tail-vein injection of CCPA (75 µg/kg) and 24 hours later the hearts were excised and Langendorff-perfused and subjected to 35 minutes lethal ischaemia and 120 minutes reperfusion;

(3) ***CCPA-Induced SWOP+Atr:*** Rats received a tail-vein injection of CCPA (75 µg/kg) and 24 hours later the hearts were excised and Langendorff-perfused and subjected to 35 minutes lethal ischaemia and 120 minutes reperfusion, and were perfused with atractyloside (20 µmol/l, a known opener of the mPTP)([8-10]) for the first 15 minutes of reperfusion. This concentration of atractyloside has been previously demonstrated to reverse the effects of calcium-preconditioning in adult rat myocytes;([251])

(4) ***Atr controls*:** Rats (n=6) received a tail-vein injection of saline vehicle and 24 hours later the hearts were excised and Langendorff-perfused and subjected to 35 minutes lethal ischaemia and 120 minutes reperfusion, and were perfused with atractyloside (20 µmol/l) for the first 15 minutes of reperfusion.

Figure 5.7: ***Experimental protocols Investigating the Effect of Opening the mPTP at the Time of Reperfusion in Hearts Treated with CCPA-Induced Second Window of Protection*** (SWOP). Atr-Atractyloside, TTC-tetrazolium, CCPA-2 chloro-N^6-cyclopentyl-adenosine.



5.5.4 Results

5.5.4.1 Exclusions

We used 24 male Sprague-Dawley rat hearts for the infarct size experiments of which 0 were excluded (see section 3.2.3 for exclusion criteria).

5.5.4.2 Animal and Haemodynamic Data

Animal and heart weights and risk zone volumes were similar in the experimental groups (see table 5.7). Baseline data relating to cardiac function (assessed by the rate-pressure-product, RPP) and coronary flow before the lethal ischaemic period, were similar in the experimental groups (see table 5.8, 5.9). During regional ischaemia, coronary flow and RPP decreased to a similar extent in both groups. An increase in cardiac function and coronary flow upon reperfusion was indicative of successful re-flow.

Table 5.7 Characteristics of Animals in Treatment Groups

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
1. Control	6	440±16	2.3±0.07	0.638±0.029
2. CCPA	6	397±12	2.1±0.02	0.615±0.026
3. CCPA + Atr	6	422±24	2.0±0.09	0.561±0.029
4. Atr	6	428±21	2.3±0.07	0.653±0.041

Values are mean±SEM. *P<0.001 compared with control.

Table 5.8 Rate Pressure Product (x 10^3mmHg/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	26.8±2.0	31.8±1.5	8.6±1.8	19.6±1.0	24.4±2.1	19.8±2.9
2. CCPA	26.6±1.6	29.1±2.3	8.2±2.2	17.9±1.7	18.2±2.7	13.8±2.4
3. CCPA + Atr	27.5±1.9	33.8±2.4	7.5±0.4	22.0±2.3	23.0±4.0	18.4±0.9
4. Atr	23.6±3.1	32.0±1.6	10.9±2.6	16.2±3.4	20.8±2.6	11.7±2.0

Values are mean±SEM. *P<0.005 compared with control.

Table 5.9 Coronary Flow Rate (ml/min)

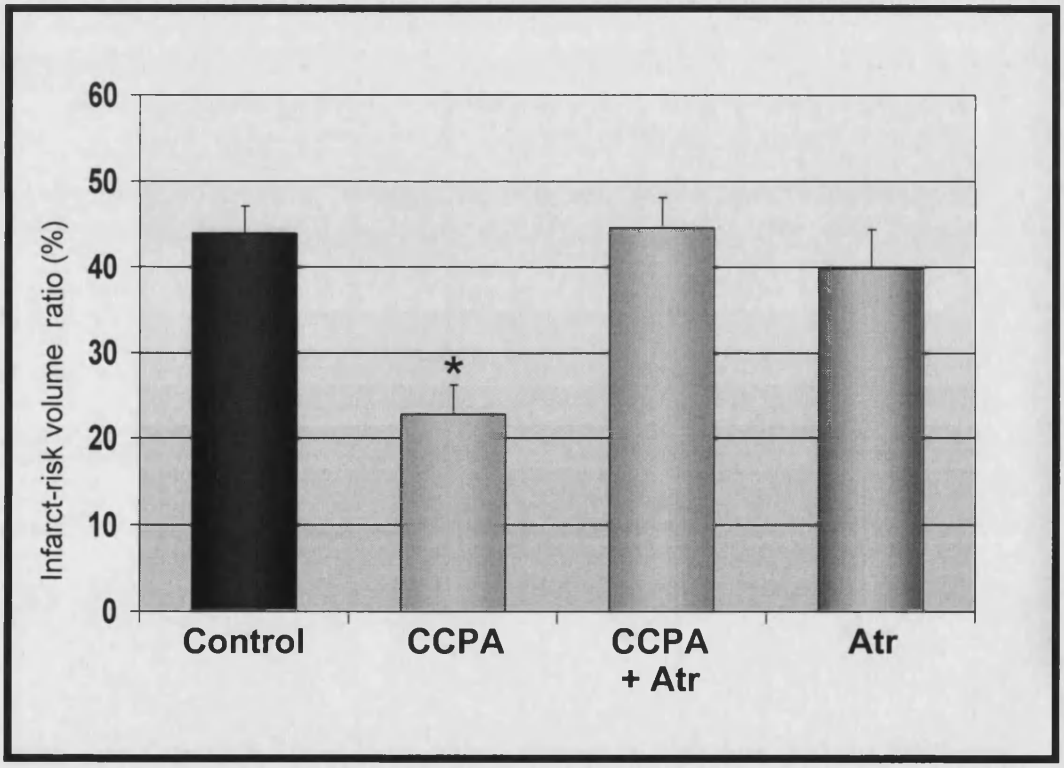
Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	21.2±1.1	21.2±1.1	10.0±0.5	9.5±0.6	16.5±1.1	12.2±0.7
2. CCPA	18.2±0.8	17.8±0.7	8.8±0.4	8.5±0.3	13.0±0.8	8.8±1.6
3. CCPA + Atr	18.0±0.8	17.6±0.9	8.2±0.6	8.8±0.7	11.0±0.9	9.4±1.1
4. Atr	18.3±0.8	18.0±0.9	9.7±0.8	9.3±0.8	14.8±1.9	9.3±1.0

Values are mean±SEM. *P<0.005 compared with control.

5.5.4.3 Infarct Size Data

Infarct size is represented as the percentage of tetrazolium-negative tissue in the ischaemic risk zone. CCPA-induced SWOP reduced infarct size from 43.9±3.1% in control to 22.7±1.9% with CCPA (P<0.001; figure 5.8). When atractyloside was administered for the first 15 minutes of reperfusion to open the mPTP, CCPA-induced protection was completely abolished (22.7±1.9% with CCPA vs 44.6±3.5% with CCPA+Atr; P<0.001; figure 5.8). Atractyloside given alone at reperfusion did not influence infarct size (39.8±4.6% with Atr vs 43.9±3.1% in control; P=NS; figure 5.8).

Figure 5.8: ***Pharmacologically Opening the mPTP at Reperfusion Abrogates the Protection Associated with CCPA-Induced SWOP.*** Pharmacologically opening the mPTP for the first 15 minutes of reperfusion using atractyloside (Atr) abrogated the protection associated with CCPA-induced second window of protection (SWOP). 2 chloro-N^6-cyclopentyl-adenosine (CCPA). *P<0.001 compared with control.



5.5.4 Discussion

In this part of the study we have shown that pharmacologically opening the mPTP at the time of reperfusion, using atractyloside, abrogates the protection associated with the second window of protection induced by the adenosine A1-receptor agonist, CCPA, suggesting that SWOP may also modulate mPTP opening. A recent study by Rajesh and colleagues using ischaemia-induced delayed preconditioning has confirmed our findings.([882]) They demonstrated, that ischaemic preconditioning protected the isolated perfused rat heart 24 hours later, as evidenced by a reduction in infarct size, increased expression of the anti-apoptotic protein Bcl-2, attenuation of apoptotic cell death and mitochondrial swelling and cytochrome C release. The mPTP openers Ionidamine or atractyloside, when given prior to the index ischaemic episode were shown to abolish the protective effect associated with ischaemic preconditioning.([882]) However, they did not demonstrate directly that the SWOP actually inhibits mPTP opening, but have implied it based on the effects of mPTP opener, much like we have done in this part of the study.

Interestingly, we did not observe an increase in infarct size with control hearts given atractyloside at the time of reperfusion, a finding supported by Rajesh and colleagues, who found no difference in infarct size with either of the mPTP openers atractyloside or Ionidamine when compared to control hearts.([882])

The findings from this section and section 5.4 provide only indirect evidence that the mPTP is modulated in myocardial preconditioning. Therefore, the next objective was to determine whether preconditioning inhibits mPTP opening directly.

5.6 Aim (4)

Determine whether preconditioning inhibits calcium-induced opening of the mPTP in isolated mitochondria

In this part of the study we examined the effect of preconditioning on mPTP opening directly. We used a method for inducing and detecting mPTP opening in adult rat mitochondria, in which mitochondria were loaded with the fluorescent, calcein. Opening of the mPTP was induced by calcium loading and was detected as a reduction in mitochondrial calcein fluorescence, which was measured using a flow cytometer. As such, the method was adapted from that used by Pastorino and colleagues([755]) who loaded mitochondria with calcein and detected the reduction in mitochondrial fluorescence due to mPTP opening, using a fluorescence spectrophotometer. As mitochondrial Ca^{2+} loading during ischaemia-reperfusion is a key determinant of mPTP opening at the time of reperfusion, this model is relevant to examining the effect of preconditioning on the mPTP opening-induced by mitochondrial Ca^{2+}. In this regard, the effect of the preconditioning-mimetics diazoxide and CCPA (the adenosine A1-receptor agonist) on Ca^{2+}-induced mPTP opening was examined.

5.6.1 Materials

Diazoxide was dissolved in dimethyl sulphoxide (DMSO, Sigma Chemicals, Poole, Dorset), giving a final concentration of <0.1% DMSO. Cyclosporin-A (Sigma Chemicals, Poole, Dorset) was dissolved in 50% ethanol, giving a final concentration of 0.05% ethanol. Atractyloside (Sigma Chemicals, Poole, Dorset), 5-Hydroxydecanoic acid (5-HD, Sigma Chemicals, Poole, Dorset) and 2 chloro-N^6-cyclopentyl-adenosine (CCPA, Sigma Chemicals, Poole, Dorset), were dissolved in distilled H_2O. The fluorescent dyes calcein-AM (calcein-acetoxymethyl ester, Molecular Probes Inc., Leiden, The Netherlands) and tetramethyl rhodamine methyl ester (TMRM, Molecular Probes Inc., Leiden, The Netherlands) were dissolved in dimethyl sulphoxide (DMSO, Sigma Chemicals, Poole, Dorset). All other reagents were of standard analytical grade.

5.6.2 Mitochondrial Model of mPTP Opening

In this study, mPTP opening was induced and detected in isolated adult rat mitochondria, which involved following the movement of a membrane-impermeant fluorescent dye, calcein-acetoxymethyl ester (calcein-AM) out of the mitochondria on mPTP opening. The reduction in mitochondrial calcein fluorescence was measured using a flow cytometer.

Mitochondria were isolated from male Sprague-Dawley rats according to the method described in section 3.3. Aliquots of the mitochondrial sample (0.5 mg/ml) were incubated with the fluorescent dye, calcein-AM (1 μmol/L) for 15 minutes at room temperature. The ester form of calcein, termed calcein-AM is membrane permeable, unlike free calcein which is membrane impermeable. Therefore calcein-AM readily enters the mitochondria and once within the mitochondrial matrix it is de-esterified (by mitochondrial esterases), releasing the free fluorescent calcein which is trapped in the mitochondrial matrix as it is membrane impermeable. Therefore, calcein (which has a molecular weight of 620 Da) can only exit the mitochondria via the mPTP, which allows solutes <1500 Da to pass through.([9;848]) Therefore, in this model mPTP opening is indicated by the reduction in calcein fluorescence, which was measured using flow cytometry (see section 3.4). For calcein-fluorescence, the signal was analysed in the FL1 detector channel equipped with a band-pass filter at 520 nm; the photo-multiplier value of the detector was 631 V. In this model, mPTP opening was induced using either calcium or atractyloside (both known inducers of mPTP opening).([8-10]) Using this model, the effect of the preconditioning-mimetic diazoxide on Ca^{2+}-induced mPTP opening was examined.

The mitochondria were then washed twice with KCl buffer containing (in mmol/l) KCl 120.0, TES 5.0, and $MgCl_2$ 0.1, with added ATP 0.2 and sodium succinate 10. To confirm that the observed reduction in mitochondrial calcein fluorescence was due to mPTP opening it was essential to demonstrate that it was sensitive to the mPTP inhibitor, cyclosporin-A.

5.6.3 Experimental Protocols for Mitochondrial Studies

Calcein-loaded mitochondria in KCl buffer were incubated for 10 minutes at room temperature with the following treatments:

(1) ***Control***: 0.1 μmol/l Ca^{2+} in the presence or absence of 0.05% ethanol or 0.1% DMSO;

(2) ***Calcium-induced mPTP opening***: 500 μmol/l Ca^{2+};

(3) ***Atractyloside-induced mPTP opening***: 0.1 μmol/l Ca^{2+} + atractyloside (5 mmol/l). This concentration of atractyloside has been used to induce mPTP opening in isolated mitochondria;([884])

(4) ***Calcium and CsA***: 500 μmol/L Ca^{2+} in the presence of cyclosporine-A (0.2 μmol/l). This concentration of CsA confers potent inhibition of mPTP opening;([780])

(4) ***Atractyloside and CsA:*** 0.1 μmol/l Ca^{2+} + atractyloside (5 mmol/l) in the presence of cyclosporine-A (0.2 μmol/l);

(5) ***Calcium and diazoxide***: 500 μmol/l Ca^{2+} in the presence of the preconditioning-mimetic diazoxide (30 μmol/l). This concentration of diazoxide has been used as a preconditioning-mimetic;([104])

(6) ***Calcium, diazoxide and 5-HD***: 500 μmol/l Ca^{2+} in the presence of diazoxide (30 μmol/l) and 5-HD (100 μmol/l). This concentration of 5-HD has been used to close the mitochondrial K_{ATP} channel;([104])

(7) ***Calcium and CCPA:*** 500 μmol/l Ca^{2+} in the presence of the adenosine A1-receptor agonist, CCPA (200 nmol/l). This concentration of CCPA has been used as a preconditioning mimetic;([880]) and

(8) ***Calcium and 5-HD***: 500 μmol/l Ca^{2+} in the presence of 5-HD (100 μmol/L).

Following incubation with the above treatments, mitochondrial calcein fluorescence was measured using the flow cytometer (see section 3.4). Experiments were performed on mitochondria isolated from 6 individual rats.

5.6.4 Results

5.6.4.1 Exclusions

We used 14 male Sprague-Dawley rat hearts for mitochondrial isolation of which 2 were excluded owing to poor mitochondrial function (see section 3.2.3 for exclusion criteria).

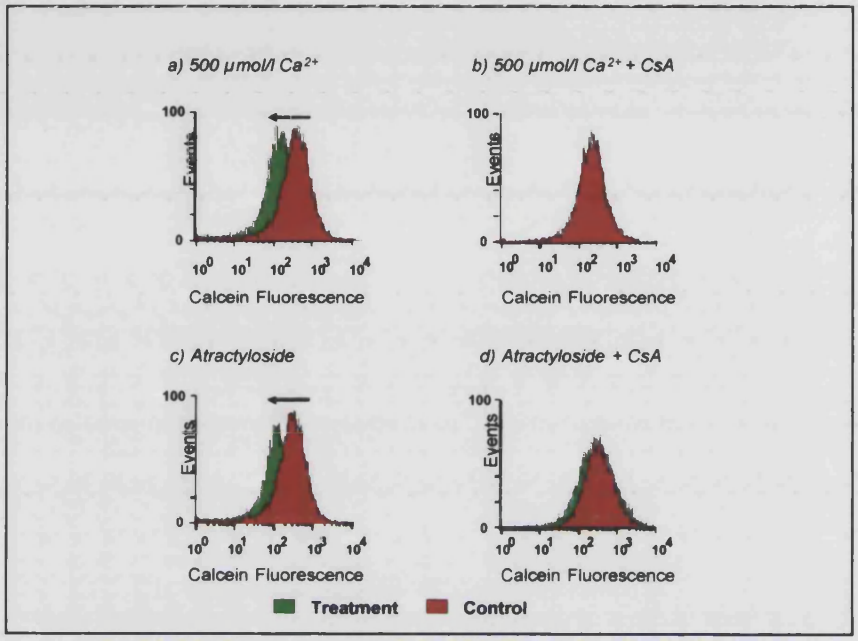
5.6.4.2 Mitochondrial Calcein Fluorescence Data

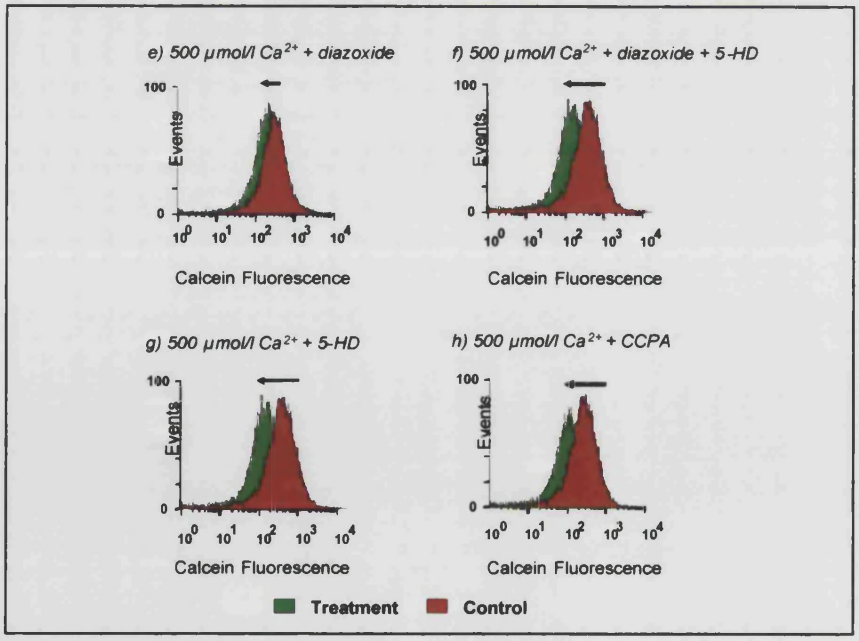
Arithmetic mean values of the median mitochondrial calcein fluorescence intensities were determined for each sample for graphic representation. Figure 5.9 (a-h) shows representative

flow cytometric profiles following various treatment protocols. The Y scale denotes the number of events which corresponds to the number of mitochondria emitting a calcein fluorescence signal portrayed on the X axis. A shift to the left in the profile signifies a reduction in the median mitochondrial fluorescence for the population of mitochondria, due to opening of the mPTP.

The mitochondrial calcein fluorescence values after treatment with Ca^{2+} (0.1 μmol/l) plus or minus the treatment drug were taken as the values from which any reduction in fluorescence was measured. Exposure of mitochondria to Ca^{2+} (500 μmol/l) induced a reduction in calcein fluorescence of 41.9±3.5% (figure 5.10a), indicating mPTP opening. This Ca^{2+}-induced reduction in calcein fluorescence was abolished in the presence of the mPTP inhibitor, cyclosporin-A (0.7±0.7% with 500 μmol/l Ca^{2+} + CsA vs 41.9±3.5% with 500 μmol/l Ca^{2+} alone, $P<0.0001$; figure 5.10a), indicating that the calcium-induced reduction in calcein fluorescence was due to opening of the mPTP. The mPTP opener, atractyloside induced a reduction in calcein fluorescence of 40.3±3.1% (figure 5.10a), indicating mPTP opening, which was also sensitive to cyclosporin-A (7.0±2.0% with atractyloside + CsA vs 40.3±3.1% with atractyloside alone, $P<0.0001$; figure 5.10a), indicating that the atractyloside-induced reduction in calcein fluorescence was due to opening of the mPTP.

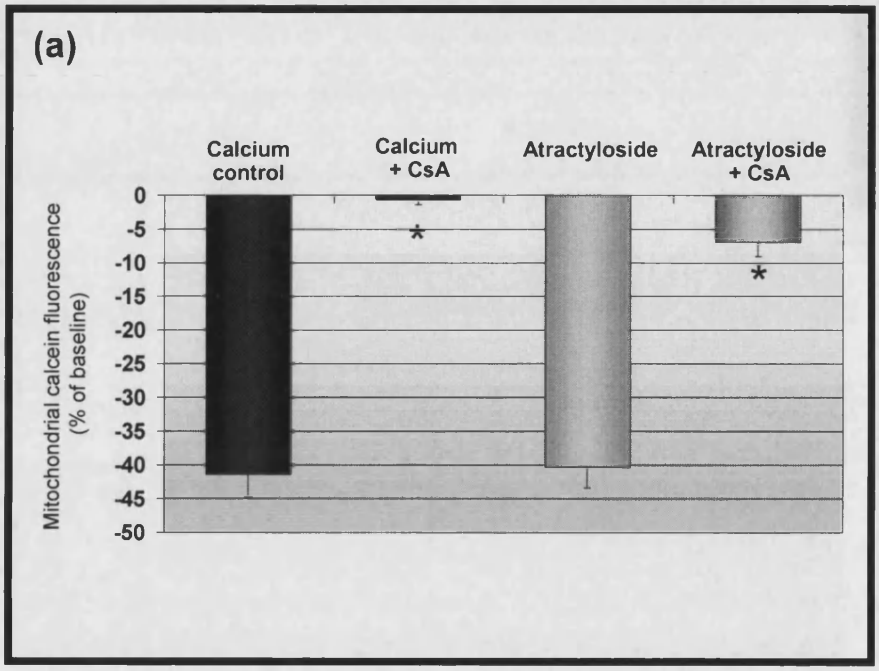
Figure 5.9 (a-h): Flow Cytometric Profiles of Mitochondrial Calcein Fluorescence. Treatment (green profiles) with either (a) 500 µmol/l Ca^{2+} or (c) Atractyloside induces a reduction in mitochondrial calcein fluorescence, indicating mPTP opening, compared to the red control profiles. The presence of the mPTP inhibitor, cyclosporin-A (CsA) prevents the reduction in calcein fluorescence induced by (b) 500 µmol/l Ca^{2+} or (d) Atractyloside, indicating inhibition of mPTP opening. The presence of the preconditioning-mimetic, diazoxide, attenuates the reduction in calcein fluorescence induced by (e) 500 µmol/l Ca^{2+}, indicating inhibition of mPTP opening. This effect of diazoxide on mPTP opening is abolished in the presence of the mitochondrial K_{ATP} channel blocker, 5-HD (f). The adenosine A1-receptor agonist, CCPA and 5HD do not have any effect on mPTP opening (h, i).

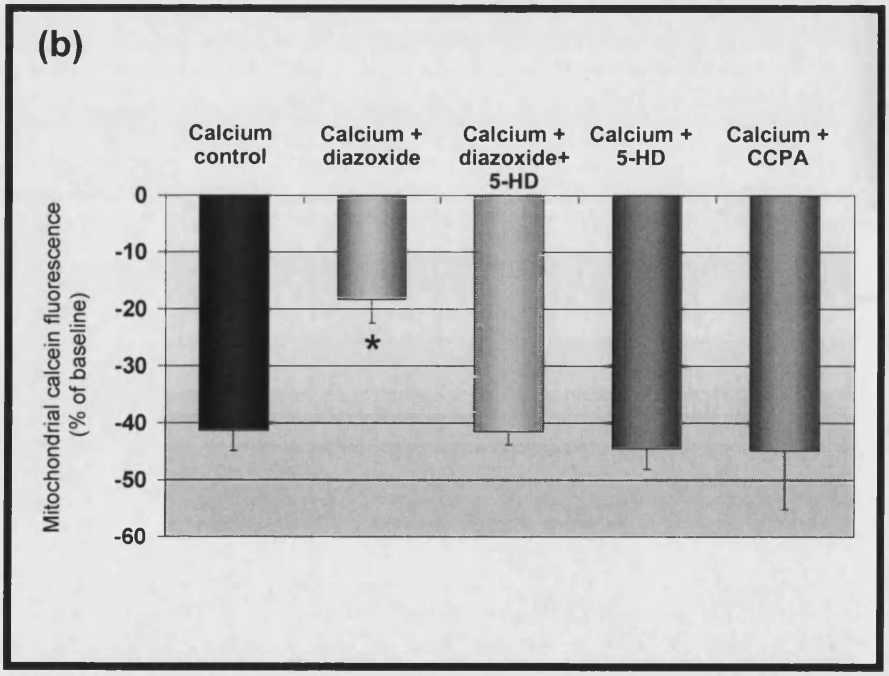




The presence of the preconditioning-mimetic, diazoxide attenuated the calcium-induced reduction in calcein fluorescence (18.4±4.1% with 500 μmol/l Ca^{2+} + diazoxide vs 41.9±3.5% with 500 μmol/l Ca^{2+} alone, P<0.0001; Figure 5.10b), which is equivalent to a 52.5±8.0% reduction, indicating that pharmacological preconditioning using diazoxide inhibits calcium-induced mPTP opening. This effect of diazoxide was abolished in the presence of the mitochondrial K_{ATP} channel blocker, 5-HD (41.5±2.4% with 500 μmol/l Ca^{2+} + diazoxide + 5-HD vs 18.4±4.1% with 500 μmol/l Ca^{2+} + diazoxide, P<0.0001; figure 5.10b). 5-HD alone did not influence the reduction in calcein fluorescence (44.5±3.7% with 500 μmol/l Ca^{2+} + 5-HD vs 41.9±3.5% with 500 μmol/L Ca^{2+} alone; figure 5.10b). CPA had no effect on the calcium-induced reduction in calcein fluorescence (44.9±10.2% vs 41.9±3.5% with 500 μmol/L Ca^{2+} alone; figure 5.10b).

Figure 5.10 (a-b): Changes in Mitochondrial Calcein Fluorescence. (a) Treatment with either 500 µmol/l Ca^{2+} or atractyloside induces a reduction in mitochondrial calcein fluorescence, indicating mPTP opening. The presence of the mPTP inhibitor, cyclosporin-A (CsA) prevents the reduction in calcein fluorescence induced by 500 µmol/l Ca^{2+} or atractyloside, indicating inhibition of mPTP opening. (b) The presence of the preconditioning-mimetic, diazoxide, attenuates the reduction in calcein fluorescence induced by 500 µmol/l Ca^{2+}, indicating inhibition of mPTP opening. This effect of diazoxide on mPTP opening is abolished in the presence of the mitochondrial K_{ATP} channel blocker, 5-HD. The adenosine A1-receptor agonist, CCPA and 5HD do not have any effect on mPTP opening. Values are means of the median mitochondrial calcein fluorescence for that group ±SEM expressed as a % of the baseline fluorescence.*P<0.0001 compared to control.





5.6.5 Discussion

The results demonstrate that the treatment with the preconditioning mimetic, diazoxide, inhibits the mPTP opening induced by mitochondrial Ca^{2+} loading. The inhibitory effect of diazoxide on mPTP opening was abolished in the presence of the mitochondrial K_{ATP} channel blocker, 5-HD, suggesting that this effect of diazoxide can be antagonised by 5-HD, whether it be due to the closing of the mitochondrial K_{ATP} channel or independent of the mitochondrial K_{ATP} channel. As expected the adenosine-A1 receptor agonist had no effect on Ca^{2+}-induced mPTP opening, since this drug exerts its effect by activating a cell-surface G-protein coupled receptor, not present in a mitochondrial preparation.

This effect of diazoxide on mPTP opening has been confirmed in a study by Weiss and colleagues,([846]) who demonstrated that diazoxide inhibited the mPTP opening induced by mitochondrial Ca^{2+} loading, using the reduction in mitochondrial calcein fluorescence to indicate mPTP opening. In addition, an agonist of PKC (implicated as a mediator of IPC) was demonstrated to inhibit Ca^{2+}-induced mPTP opening in their model.([846]) However, in their study they used a very high concentration of calcein of 10 µmol/l, which was ten times greater than the concentration used in our study. At this higher concentration, calcein would be expected to generate mitochondrial oxidative stress.([885;886])

The results from this part of the study suggests that the preconditioning mimetic diazoxide may be able to protect the heart by inhibiting the Ca^{2+}-induced mPTP opening that occurs in the setting of ischaemia-reperfusion. However, the mechanism by which diazoxide inhibits Ca^{2+}-induced mPTP opening is unclear, though it could relate to the effect of diazoxide on mitochondrial [Ca^{2+}]. The mitochondrial entry of Ca^{2+} into occurs via the Ca^{2+}-uniporter and is determined by the mitochondrial membrane potential. Several studies have suggested diazoxide can attenuate mitochondrial Ca^{2+} loading by reducing the mitochondrial membrane potential. ([257;258]) However, these studies have attracted controversy because of the high concentrations of diazoxide and pinacidil used and over whether mitochondrial K_{ATP} channel opening actually induces a significant mitochondrial membrane depolarisation.([109;159;160;259]) At high concentrations, diazoxide and pinacidil can uncouple mitochondria, independent of mitochondrial K_{ATP} channel opening.([160;260]) Subsequent studies in other laboratories however, have noted that diazoxide can protect against ischaemia-reperfusion injury by causing reduction in mitochondrial Ca^{2+} load;([52;239;261;262])

It may well transpire that the conditions in which diazoxide induces a significant mitochondrial membrane depolarisation are: (1) if high concentrations of diazoxide (>100 μmol/l) are used in which case uncoupling occurs independent of the mitochondrial K_{ATP} channel;([160;260]) or (2) if the mitochondria are de-energised, such that a K^+ influx induced by diazoxide is sufficient to uncouple the already compromised mitochondrial respiratory function. This is supported by a study by Weiss' group who demonstrated significant diazoxide-induced mitochondrial membrane depolarisation in de-energised but not energised mitochondria.([846]) Further evidence is provided by the revelation that the diazoxide-induced flavoprotein oxidation (secondary to mitochondrial uncoupling), used by Marban's group to indicate opening of the mitochondrial K_{ATP} channel, was only observed in de-energised cells-i.e. cells which had been kept in substrate-free medium overnight.([108;110])

Another major determinant of mPTP opening in the setting of ischaemia-reperfusion is oxidative stress, which is generated on the reperfusion of ischaemic myocardium.([79;276;277]) Therefore, in the next section of the study, we investigated the effects of preconditioning on the mPTP opening induced by oxidative stress.

5.7 Aim (5)

Determine whether preconditioning inhibits oxidative stress-induced opening of the mPTP in isolated myocytes

Having demonstrated in the previous section that the preconditioning mimetic, diazoxide can inhibit mPTP opening induced by mitochondrial Ca^{2+} loading, our next objective was to determine whether preconditioning could inhibit the mPTP opening induced by oxidative stress. Together with the mitochondrial [Ca^{2+}], oxidative stress is a major determinant of mPTP opening at the time of reperfusion. Using a model which allowed us to induce and detect mPTP opening both accurately and reproducibly, we investigated the effects of hypoxic preconditioning (HP) and pharmacological preconditioning using diazoxide or nicorandil on the mPTP opening induced by oxidative stress.

5.7.1 Materials

Diazoxide (Sigma Chemicals, Poole, Dorset), glibenclamide (Sigma Chemicals, Poole, Dorset) and sanglifehrin-A (SfA, Novartis Pharm AG, Basel, Switzerland) were dissolved in dimethylsulphoxide (DMSO), giving a final concentration of <0.1% DMSO. Cyclosporin-A (CsA, Sigma Chemicals, Poole, Dorset) and N-methyl 4-valine CsA (Novartis Pharm AG, Basel, Switzerland) were dissolved in 50% ethanol, giving a final concentration of 0.05% ethanol. HMR 1098 (gift of Dr J. Downey) and 5-hydroxydecanoate (5-HD, Sigma Chemicals, Poole, Dorset) were dissolved in distilled water. Nicorandil (Nic, Chugai Pharmaceuticals, Tokyo, Japan) was dissolved in normal saline. Tetramethylrhodamine methyl ester (TMRM, Molecular Probes Europe BV, Leiden, The Netherlands) was dissolved in DMSO. All other agents were of standard analytical grade and quality.

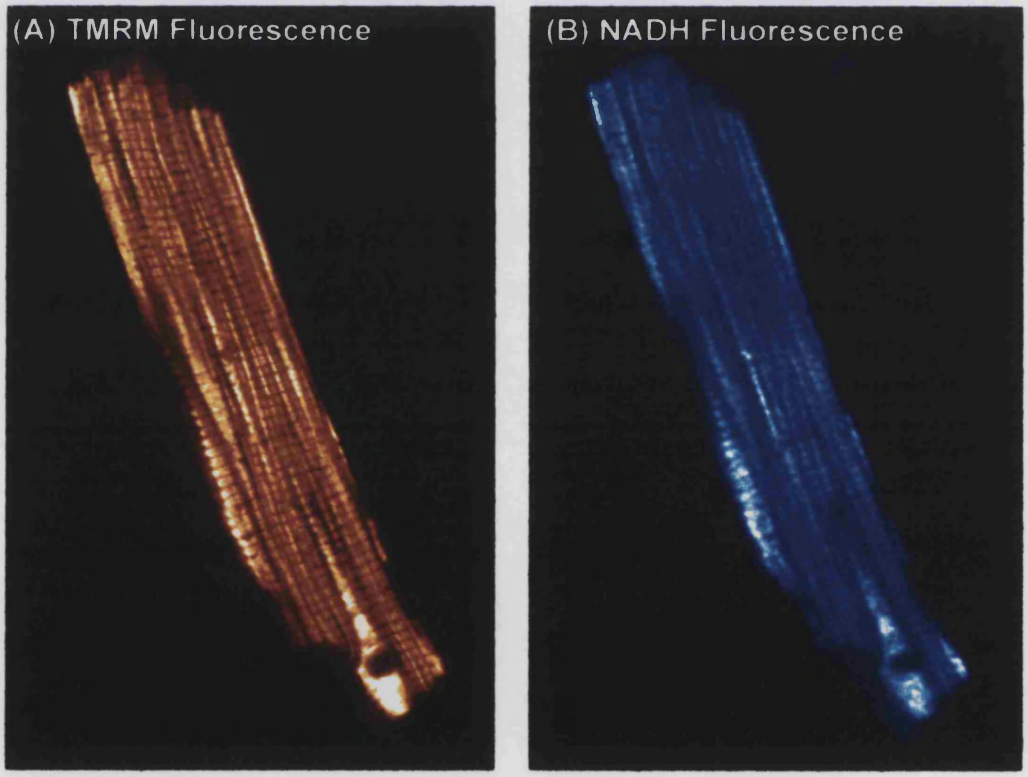
5.7.2 Myocyte Model of mPTP Opening

Adult rat myocytes were isolated from male Sprague-Dawley rats according to the method in described in section 3.5. Isolated myocytes were seeded onto 25-mm round cover-slips according to the method in described in section 3.6.

Opening of the mPTP in adult rat myocytes was induced and detected using a cellular model of oxidative stress.([647;768;827-830]) Seeded myocytes, in restoration buffer, were incubated with the fluorescent dye, TMRM (3 μmol/l) for 15 minutes at 37°C, washed and visualised using confocal fluorescence microscopy, as described in section 3.7.

TMRM, a lipophilic cation, accumulates selectively into mitochondria according to the mitochondrial membrane potential([845]) (figure 5.11 shows an adult rat myocyte loaded with TMRM). In this figure, NADH fluorescence (representing autofluorescence) is also shown to demonstrate the co-localisation of the fluorescence signal, verifying that the TMRM localises to the mitochondria.

Figure 5.11: TMRM and NADH Fluorescence of an Adult Rat Myocyte. Representative image of adult rat myocytes demonstrating: (a) TMRM fluorescence localised to the mitochondria as bands of yellow-orange fluorescence (b) NADH fluorescence indicating mitochondrial autofluorescence as bands of blue fluorescence. Note how the TMRM and NADH fluorescent signals are co-localised to the mitochondria, confirming that TMRM localises to the mitochondria.



Laser-illumination of the TMRM dye contained within the mitochondria, photosensitises the TMRM, generating reactive oxygen species (ROS) from within the mitochondria,([887]) which induce opening of the mPTP. Opening of the mPTP, permeates the normally impermeable inner

mitochondrial membrane, which results in uncoupling of oxidative phosphorylation and subsequent collapse of the mitochondrial membrane potential. In this model, the opening of the mPTP is represented by the collapse in mitochondrial membrane potential.([825;829]) Oxidative stress generated on reperfusing ischaemic myocardium also involves the excess production of ROS from within the mitochondria,([276;277;450]) and this model therefore simulates the events associated with lethal reperfusion injury. This model represents a widely reported and reliable way to reproducibly induce the loss of mitochondrial membrane potential, which has been unequivocally identified as mPTP opening.([768;818;825;827-830])

The relatively high concentration of TMRM in the mitochondria causes auto-quenching of fluorescence, such that the fluorescence signal becomes a non-linear function of dye concentration; therefore in this model, mitochondrial membrane depolarisation results in the loss of dye into the cytosol where the signal *increases*.([887;888]) Laser-induced oxidative stress was applied until mPTP opening had been induced (indicated by collapse of the mitochondrial potential) and continued until rigour contracture occurred. We measured the duration of time taken to induce mPTP opening, which corresponds to the burden of ROS required to induce mPTP opening. Following the induction of mPTP opening, the additional duration of time taken to induce rigour contracture (signalling ATP depletion) was also measured.

For all photo-sensitisation experiments, the conditions of the confocal imaging system (laser power, confocal pinhole - set to give an optical slice of 1 micron - pixel dwell time, and detector sensitivity) were identical to ensure comparability between experiments.

5.7.3 Experimental Protocols for Myocyte Studies

After loading with TMRM in the restoration buffer, cells were randomly assigned to one of the following treatment groups. Cells were incubated for 20 minutes with the drug(s) at 37°C and were then subjected to the TMRM-oxidative stress protocol.

1 ***Control***-incubation with restoration buffer alone (n=18), with 0.05% ethanol (n=6), or 0.1% DMSO vehicle control (n=6);

2 ***Cyclosporin-A*** (0.2 μmol/l, an inhibitor of mPTP opening, n=12);

3 ***N-methyl 4-valine-CsA*** (0.4 μmol/l, an inhibitor of mPTP opening, n=12). This concentration of N-methyl 4-valine-CsA has been use to inhibit mPTP opening;([830])

4 ***Sangliefehrin-A*** (1.0 μmol/l, n=12). This concentration of sangliefehrin-A has been demonstrated to be a potent mPTP inhibitor;([676])

5 ***Hypoxic preconditioning*** (HP) (n=12)-see section 5.7.4;

6 ***HP + 5-HD***: HP in the presence of 5-HD (100 μmol/l, a mitochondrial K_{ATP} channel blocker, n=6). This concentration of 5-HD has been shown to block the mitochondrial K_{ATP} channel;([104])

7 ***HP + glibenclamide***: HP in the presence of glibenclamide (10 μM, n=6). This concentration of glibenclamide has been shown to block the mitochondrial K_{ATP} channel;([875])

8 ***Diazoxide*** (30 μmol/l, n=12). After incubation of the cells with diazoxide the cells were washed with restoration buffer prior to the oxidative stress protocol. This concentration of diazoxide has been shown to act as a preconditioning mimetic;([104])

9 ***Diazoxide + 5-HD*** (n=6);

10 ***Diazoxide + glibenclamide*** (n=6);

11 ***Diazoxide + HMR 1098*** (10 μmol/l, n=6).

12 ***Nicorandil*** (100 μmol/l, n=12). After incubation of the cells with nicorandil the cells were washed with restoration buffer prior to the oxidative stress protocol. This concentration of nicorandil has been shown to act as a preconditioning mimetic;([889])

13 ***Nicorandil + 5-HD*** (n=6);

14 ***Nicorandil + glibenclamide*** (n=6);

15 ***5-HD*** alone (n=6);

16 ***Glibenclamide*** alone (n=6);

17 ***HMR 1098*** alone (n=6).

5.7.4 Hypoxic Preconditioning Protocol

Cells were incubated in a hypoxic (PaO_2 < 3 KPa) incubator (Heraeus, Kendro Laboratory Products, Germany) for 2 periods of 10 minutes at 37°C in anoxic buffer comprising (in mmol/l): 137.0 NaCl, 12.0 KCl, 0.49 $MgCl_2$, 4.0 HEPES, 0.9 $CaCl_2$, 1.0 Na dithionite, 20.0 2-deoxyglucose, 20.0 lactate (pH 6.5) with an intervening 30 minutes reoxygenation in restoration buffer prior to undergoing the TMRM-induced oxidative stress protocol. The anoxic buffer is a modification of that described by Esumi and colleagues.([890])

5.7.5 Results

5.7.4.1 Exclusions

We used 25 male Sprague-Dawley rat hearts for myocyte isolation of which 3 were excluded owing to poor myocyte isolation (see section 3.5 for exclusion criteria).

5.7.4.2 Myocyte Model of mPTP Opening

Confocal fluorescence imaging of adult rat ventricular myocytes loaded with TMRM, revealed mitochondria as fluorescent bands orientated with the longitudinal axis of the cell (see figure 5.12a). TMRM localises selectively to the mitochondria according to the mitochondrial membrane potential. Figure 5.12 (a-e) shows representative images extracted from a time sequence in which a myocyte was loaded with TMRM and subjected to laser-induced oxidative stress, and demonstrates the sequential changes that take place in mitochondrial membrane potential over time. Laser illumination induces firstly occasional and reversible local depolarisation of either clusters of mitochondria or individual mitochondria (which appear as areas in which TMRM fluorescence suddenly disappears, see arrows in figure 5.12b). With continued laser-induced oxidative stress, global mitochondrial membrane depolarisation occurs, seen as a 'wave' of increased TMRM fluorescence which propagates from one end of the cell to the other (a process which takes about 100 seconds), reflecting progression of irreversible mPTP opening (see arrow, figure 5.12c and figure 5.13a,b), until all the mitochondria have undergone mPTP opening (figure 5.12d). Following the depolarisation, mitochondria consume ATP ultimately leading to rigour contracture (figure 5.12e).

Figure 5.12: Confocal Images of TMRM-loaded Myocytes. Confocal fluorescent images are extracted from two separate time sequences in which a control myocyte (images on left) and a diazoxide-preconditioned myocyte (images on right) were subjected to laser-induced oxidative stress. They demonstrate the typical changes in mitochondrial membrane potential that occur in response to oxidative stress: (a) Prior to oxidative stress. (b) Localised areas devoid of TMRM fluorescence appear (see arrows). (c) A 'wave' of global mitochondrial membrane depolarisation (which indicates opening of the mPTP) begins at one end of the myocyte (see arrows), and propagates across the whole cell as a 'wave' of increase fluorescence. (d) The whole myocyte has now undergone global mitochondrial depolarisation (as mPTP opening has taken place in all the mitochondria). (e) Following the opening of the mPTP and the resultant collapse in mitochondrial membrane potential, the uncoupling of oxidative phosphorylation leads to ATP depletion and rigour contracture. Note that the time taken to induce mPTP opening and rigour contracture is increased in the diazoxide -preconditioned cell.

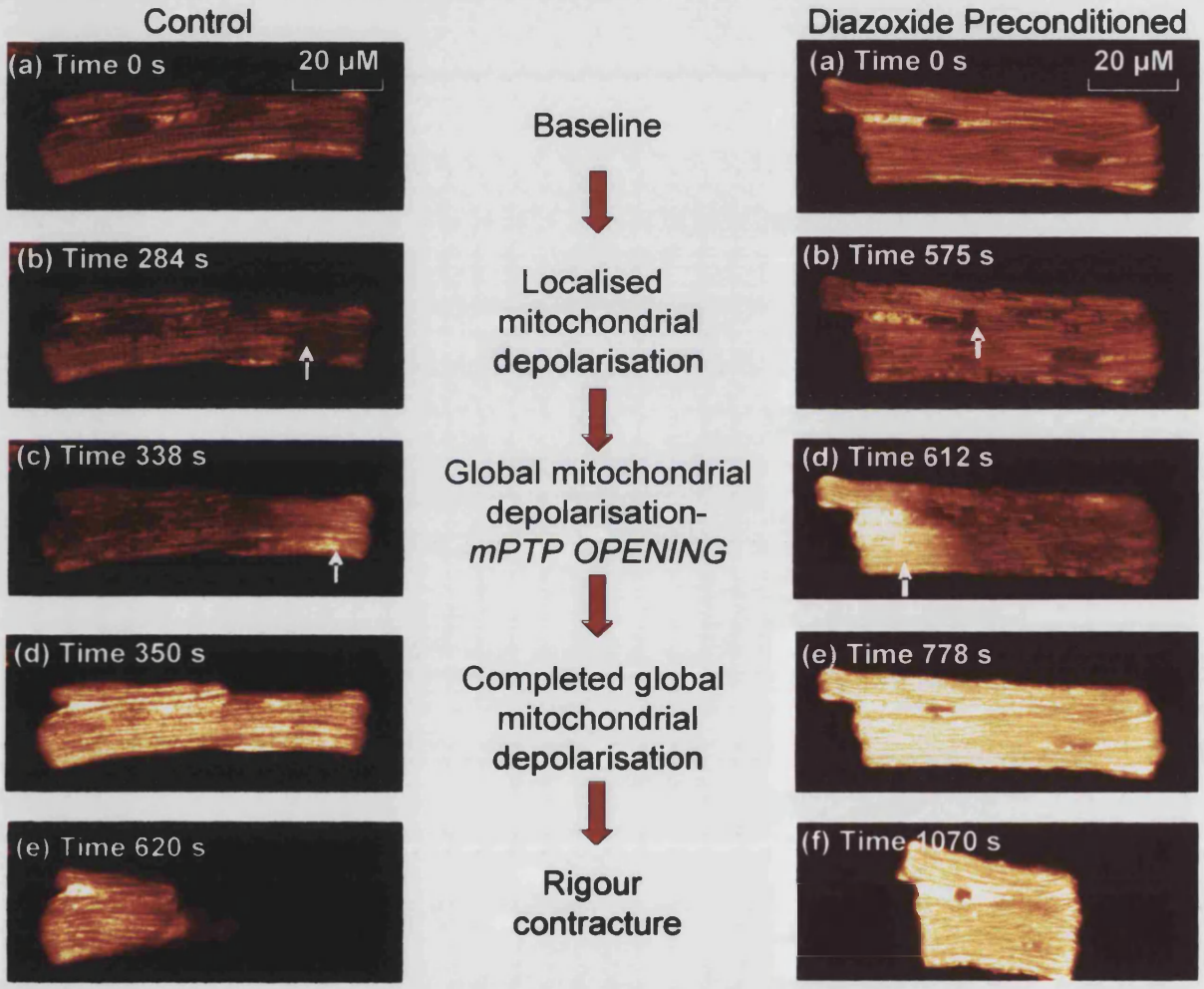
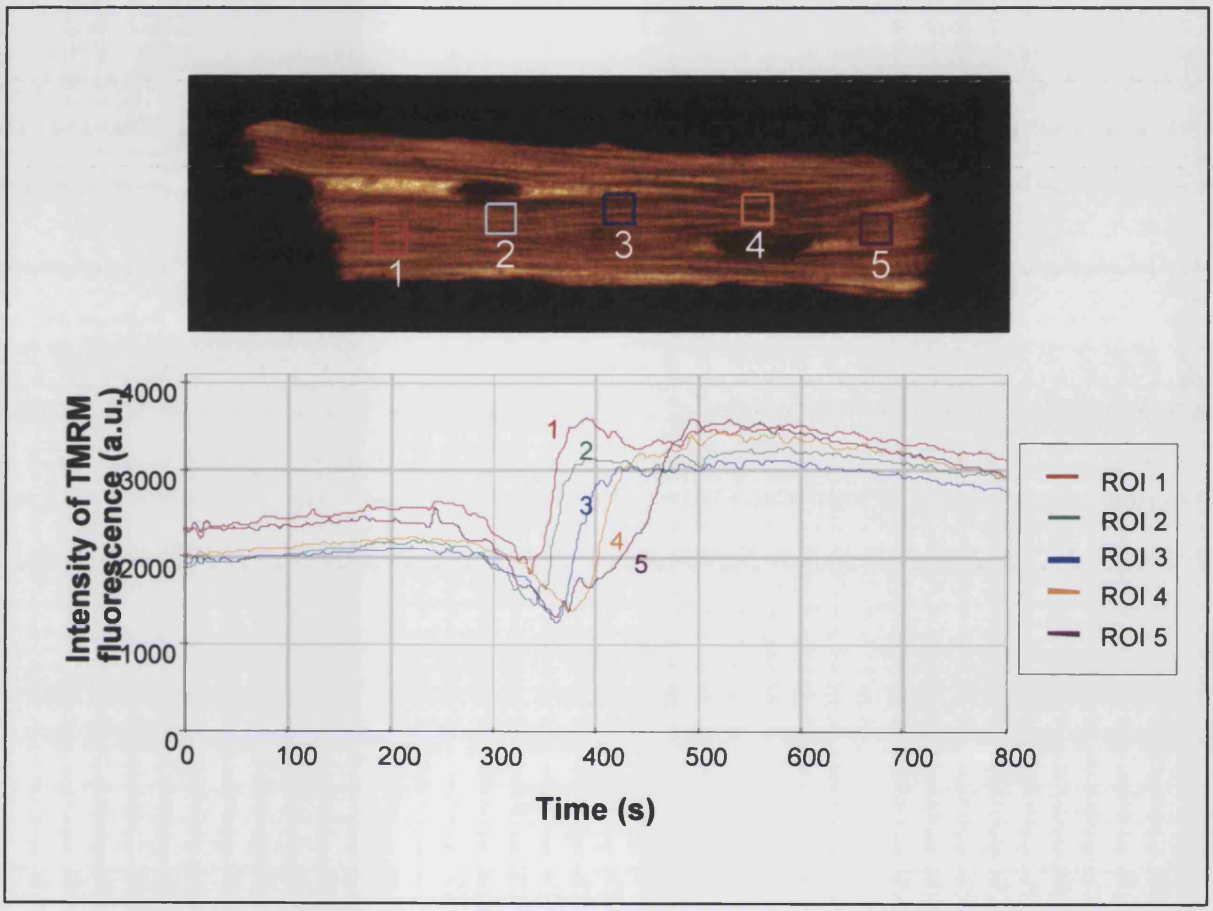


Figure 5.13: The Wave of Mitochondrial Depolarisation. (a) Representative confocal fluorescence image of a myocyte loaded with TMRM showing the 'wave' of global mitochondrial depolarisation (indicating the opening of the mPTP), demonstrated by a 'wave' of increased TMRM fluorescence propagating across the cell from left to right across five regions of interest (ROI). (b) Graph showing intensity of TMRM fluorescence in arbitrary units (a.u.) in the 5 regions of interest over time.



The times taken to induce global mitochondrial membrane depolarisation and rigour contracture were noted and in the control group, mPTP opening was routinely induced after 252.0±18.1 seconds of laser-induced oxidative stress (see figure 5.14a). A further 384 seconds were required for the progression to irreversible contraction, with rigour contracture occurring after a total of 636.1±26.4 seconds of laser-induced oxidative stress (figure 5.14b).

To verify that the observed global mitochondrial membrane depolarisation induced by laser induced oxidative stress, actually represents opening of the mPTP, it is essential to demonstrate that it is sensitive to cyclosporin-A (CsA), the most reliable inhibitor of mPTP opening.([664]) CsA was shown to extend the time required to induce opening of the mPTP more than two-fold, from 252.0±18.1 seconds to 450.7±26.1 seconds ($P<0.001$, figures 5.14b) and it also extended the time required to induce rigour contracture from 636.1±26.4 seconds to 945.4±69.7 seconds, ($P<0.001$, figures 5.14b). However, as well as inhibiting mPTP opening, CsA inhibits the phosphatase, calcineurin. To exclude the effect of CsA-induced inhibition of calcineurin in this model, we tested the non-immunosuppressive CsA analogue, N-methyl 4-valine CsA, as this drug has been shown to inhibit mPTP opening without inhibiting calcineurin.([674]) This mPTP inhibitor also increased the time taken to induce both mPTP opening and rigour contracture to 605.8±74.5 seconds and 1055.3±79.4 seconds, respectively ($P<0.001$, figure 5.14a,b). Furthermore, in this model, N-methyl 4-valine CsA was shown to be a more potent inhibitor of mPTP opening than CsA, extending the time taken to induce mPTP opening even further than CsA (605.8±74.5 seconds with N-methyl 4-valine CsA vs 450.7±26.1 with CsA, $P<0.005$, figure 5.14a), although there was no significant difference with respect to the times taken to induce rigour contracture (figure 5.14b).

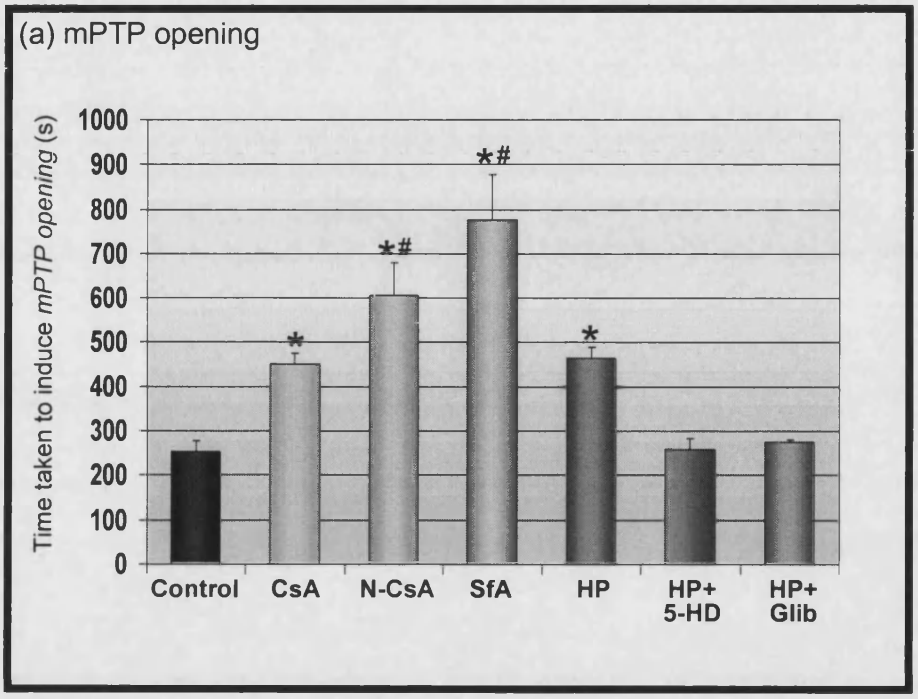
The recently described mPTP inhibitor, sanglifehrin-A,([676]) which does not inhibit calcineurin, also significantly prolonged the time taken to induce both mPTP opening and rigour contracture to 777.4±100.7 and 1329.0±129.0 seconds ($P<0.001$) respectively (figure 5.14a,b). Furthermore, sanglifehrin-A was shown to be a more potent inhibitor of mPTP opening than CsA, extending the time taken to induce mPTP opening even further than CsA (777.4±100.7 seconds with sanglifehrin-A vs 450.7±26.1 with CsA; $P<0.005$; figure 5.14a), and extending the time taken to induce rigour contracture even further than CsA (1329.0±129.0 seconds with sanglifehrin-A vs 945.4±69.7 seconds with CsA; $P<0.005$; figure 5.14b).

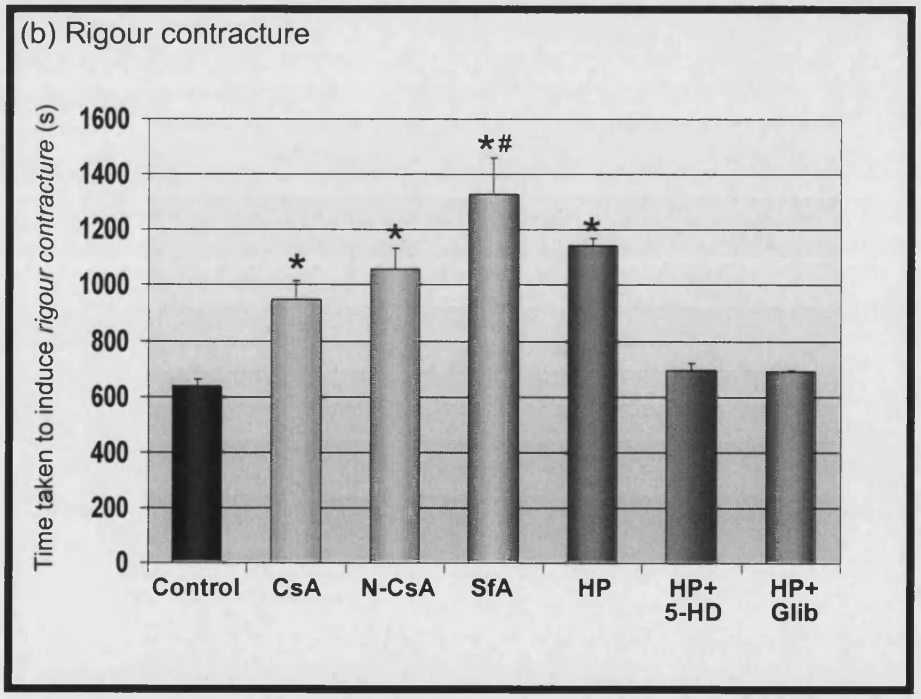
5.7.4.3 Hypoxic Preconditioning Protects the Myocyte from Oxidative Stress by Inhibiting mPTP Opening

Hypoxic preconditioning increased the illumination time required to induce global mitochondrial membrane depolarisation from 252.0±18.1 seconds in control to 464.1±53.8 seconds in hypoxic preconditioned cells, ($P<0.001$, figure 5.14a). The times taken to induce rigour was also extended from 636.1±26.4 seconds in control to 1140.7±84.0 seconds ($P<0.001$, figure 5.14b). These results suggest that hypoxic preconditioning acts to inhibit the opening of the mPTP induced by oxidative stress, resulting in protection from oxidative stress-induced rigour contracture.

The mitochondrial K_{ATP} channel blockers, glibenclamide and 5-HD abrogated the inhibitory effect of hypoxic preconditioning on mPTP opening and abolished the protective effect of preconditioning. HP in the presence of either glibenclamide or 5-HD abolished both the delay in the time taken to induce mPTP opening (464.1±53.8 seconds with HP vs 256.6±27.0 seconds with HP+glibenclamide and 247.4±7.0 seconds with HP+5-HD; $P<0.001$; figure 5.14a) and the delay in the time taken to induce rigour contracture (1140.7±84.0 seconds with HP vs 692.3±68.0 seconds with HP+glibenclamide and 687.8±30.1 seconds with HP+5-HD; $P<0.001$; figure 5.14b).

Figure 5.14: Effect of Hypoxic Preconditioning on mPTP Opening. Hypoxic preconditioning (HP) prolonged the duration of laser-induced oxidative stress required to cause both (a) mPTP opening and (b) rigour contracture in TMRM-loaded myocytes, an effect abolished in the presence of the mitochondrial K_{ATP} channel blockers, 5-hydroxydecanoate (5-HD) and glibenclamide (Glib). The m PTP inhibitors, cyclosporin-A (CsA), N-methyl 4-valine CsA and sanglifehrin-A (SfA), also prolonged the duration of laser-induced oxidative stress required to cause both (a) mPTP opening and (b) rigour contracture. (Values are Mean±SEM. *P<0.001 compared to control. #P<0.005 compared to CsA)





5.7.4.4 Pharmacological Preconditioning Protects the Myocyte From Oxidative Stress by Inhibiting mPTP Opening

Pharmacological preconditioning using either diazoxide or nicorandil also increased the illumination time required to induce global mitochondrial membrane depolarisation from 252.0±18.1 seconds in control to 528.5±28.5 seconds after diazoxide treatment, and 386.1±33.4 seconds after nicorandil treatment (P<0.001, 5.15a). The times taken to induce rigour were also extended from 636.1±26.4 seconds in control to 1095.6±102.4 seconds, and 967.6±61.8 seconds, respectively (P<0.001, 5.15b). These results suggest that pharmacological preconditioning using either diazoxide or nicorandil act to inhibit the opening of the mPTP induced by oxidative stress, resulting in protection from oxidative stress-induced rigour contracture.

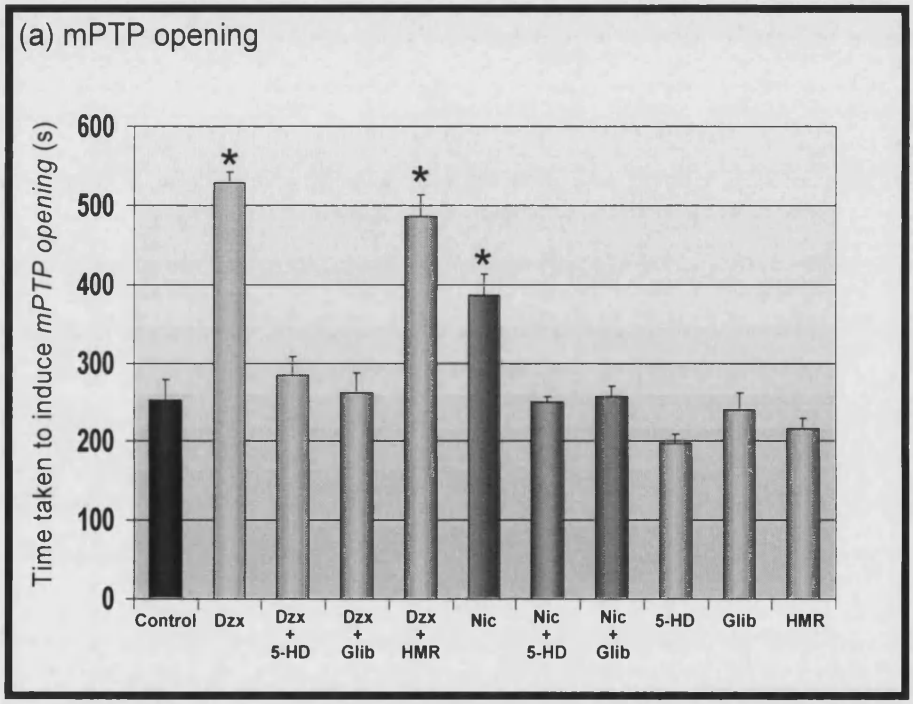
The mitochondrial K_{ATP} channel blockers, glibenclamide and 5-HD abrogated the inhibitory effect of pharmacological preconditioning on mPTP opening and abolished the protective effect of preconditioning. Therefore, diazoxide in the presence of either glibenclamide or 5-HD abolished both the delay in the time taken to induce mPTP opening (528.3±28.5 seconds with diazoxide vs 261.7±24.1 seconds with diazoxide+glibenclamide and 283.3±14.4 seconds with diazoxide+5-HD; P<0.001; figure 5.15a) and the delay in the time taken to induce rigour contracture (1095.6±102.4 seconds with diazoxide vs 543.4±47.1 seconds with diazoxide+glibenclamide and 664.0±48.3 seconds with diazoxide+5-HD; P<0.001; figure 5.15b). Furthermore, nicorandil in the presence of either glibenclamide or 5-HD also abolished both the delay in the time taken to induce mPTP opening (386.1±33.4 seconds with nicorandil vs 256.1±25.6 seconds with nicorandil+glibenclamide and 249.1±36.1 seconds with nicorandil+5-HD; P<0.001; figure 5.15a) and the delay in the time taken to induce rigour contracture (967.6±61.8 seconds with nicorandil vs 482.3±48.1 seconds with nicorandil+glibenclamide and 661.0±77.4 seconds with nicorandil+5-HD; P<0.001; figure 5.15b).

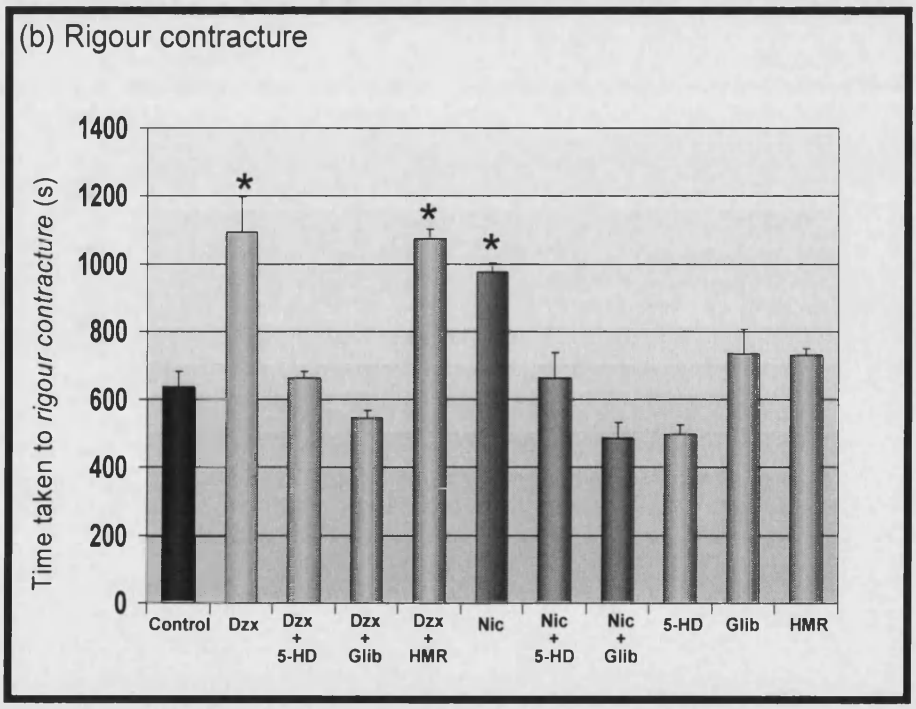
However, the specific sarcolemmal K_{ATP} channel blocker, HMR 1098, did not abolish the effects of diazoxide on either the time taken to induce mPTP opening (528.5±28.5 seconds with diazoxide vs 485.9±11.5 seconds with diazoxide+HMR 1098; P=NS; figure 5.15a) or the time taken to induce rigour contracture (1095.6±102.4 seconds with diazoxide vs 1076.8±30.3 seconds with diazoxide+HMR 1098; P=NS; figure 5.15b).

Given alone the K_{ATP} channel blockers did not influence either the time required to induce mPTP opening (252.0±18.1 seconds in control vs 239.4±22.3 seconds with

glibenclamide, 197.3±11.2 seconds with 5-HD and 216.3±13.5 seconds with HMR 1098; P=NS; figure 5.15a) or the time taken to induce rigour contracture (636.1±26.4 seconds in control vs 732.6±77.3 seconds with glibenclamide, 496.4±28.2 seconds with 5-HD and 729.4±18.8 seconds with HMR 1098; P=NS; figure 5.15b).

Figure 5.15: Effect of Pharmacological Preconditioning on mPTP Opening. Pharmacological preconditioning using either diazoxide (Dzx) or nicorandil (Nic) prolonged the duration of laser-induced oxidative stress required to cause both (a) mPTP opening and (b) rigour contracture in TMRM-loaded myocytes, an effect abolished in the presence of the mitochondrial K_{ATP} channel blockers, 5-hydroxydecanoate (5-HD) and glibenclamide (Glib) but not the sarcolemmal K_{ATP} channel blocker, HMR 1098 (HMR). The K_{ATP} channel blockers, 5-hydroxydecanoate, glibenclamide, and HMR 1098 had no effect on the time taken to induce either (a) mPTP opening or (b) rigour contracture. (Values are Mean±SEM. *P<0.001)





5.7.6 Discussion

This study shows for the first time that both hypoxic and pharmacological preconditioning, using diazoxide and nicorandil, protect the myocyte against oxidative stress by suppressing reactive oxygen species (ROS)-induced mPTP opening. Myocytes subjected to preconditioning were demonstrated to be more resistant to laser-induced oxidative stress, indicated by the fact that preconditioning treatment increased the threshold of ROS required to induce mPTP opening. The prolonged time taken to induce rigour contracture in preconditioned cells was shown to relate directly to suppression of mPTP opening, confirming the importance of the latter, in mediating cellular protection.

5.7.6.1 The Model for mPTP Opening

We used an established model for both inducing and detecting mPTP opening in the intact cell, in which laser-illumination of TMRM generates oxidative stress from within mitochondria, inducing mPTP opening, which is detected by global mitochondrial membrane depolarisation.([768;818;825;827-830]) Following the induction of mPTP opening, continued oxidative stress results in ATP depletion indicated by rigour contracture. The laser-induced collapse in mitochondrial membrane potential, as detected by increased TMRM fluorescence, was demonstrated to be sensitive to the potent mPTP inhibitor, cyclosporin-A (CsA),([664]) validating the use of this model for assessing mPTP opening in the intact cell. To exclude the effect of CsA-mediated inhibition of calcineurin, we also demonstrated that the observed mitochondrial membrane depolarisation was sensitive to the CsA analogue, N-methyl 4-valine CsA, an agent which inhibits mPTP opening without affecting calcineurin.([674]) Furthermore, the newly described mPTP inhibitor, sanglifehrin-A,([676]) which does not inhibit calcineurin, was also demonstrated to prolong the time taken to induce both mPTP opening and rigour contracture.

In the current model used to investigate mPTP opening, oxidative stress was continued until mPTP opening was induced in both control and CsA-treated cells. One would not expect CsA to completely suppress mPTP opening under these extreme conditions of oxidative stress and presumed mitochondrial Ca^{2+} overload, especially since oxidative stress can induce direct mitochondrial membrane damage by lipid peroxidation. If we had exposed control and CsA-treated cells to equal burdens of oxidative stress, it would be clear that under these conditions, CsA inhibits mPTP opening compared to the control group. From the current data it is clear that

CsA increases the tolerance to oxidative stress compared to control suggesting that CsA is inhibiting mPTP opening in this model.

The reperfusion of ischaemic myocardium, re-oxygenates the reduced mitochondrial respiratory chain,([276]) generating ROS, which in turn induces opening of the mPTP.([453]) Therefore, by contributing to the induction of mPTP opening in the first few minutes of reperfusion, ROS assume a pivotal role in mediating reperfusion-induced cell death. It was for this reason, that we investigated whether preconditioning protects the heart against ischemia-reperfusion injury by suppressing the mPTP opening that occurs in response to the oxidative stress, that is generated in the first few minutes of post-ischemic reperfusion. The findings of the current study substantiated the role of oxidative stress in mediating irreversible myocyte injury and demonstrated that preconditioning can protect the myocyte by inhibiting the mPTP opening that would ordinarily occur in response to oxidative stress.

5.7.6.2 The Role of the Mitochondrial K_{ATP} Channel

The effects of hypoxic and pharmacological preconditioning (using diazoxide or nicorandil, purported openers of the mitochondrial K_{ATP} channel) on suppressing mPTP opening and protecting against rigour contracture in the face of oxidative stress, were abrogated in the presence of 5-HD and glibenclamide, agents that have been shown to antagonize the putative mitochondrial K_{ATP} channel.([104]) This finding suggests that the observed effect of preconditioning on mPTP opening may be mediated via activation of the mitochondrial K_{ATP} channel, although controversy surrounds the nature of protection associated with agents such as diazoxide, which are reported to act via the mitochondrial K_{ATP} channel, in that they have been demonstrated to exert non-specific effects on mitochondrial function which include inhibiting mitochondrial respiration.([107;148;149;161])

5.7.6.3 Other Studies

The results of our study have been recently confirmed by a study from Marban's group, in which they demonstrated that diazoxide, nicorandil or pinacidil delayed the mitochondrial membrane depolarisation (signifying mPTP opening) induced by exogenous hydrogen peroxide, in neonatal rat myocytes.([859]) In our study we examined the effect of hypoxic preconditioning in addition to diazoxide and nicorandil on mPTP opening. Furthermore, in our model the oxidative

stress used to induce mPTP opening, was generated within mitochondria, which reproduces more closely the situation that occurs at time of reperfusion.

5.7.6.4 The 'Wave' of Mitochondrial Depolarisation

Interestingly, the ROS-induced opening of the mPTP, which was represented as a collapse in mitochondrial membrane potential, occurred as a 'wave' of mitochondrial membrane depolarisation, travelling from one end of the cell to the other in about 100 seconds. The basis for the propagation of this 'wave' of global mitochondrial depolarisation is not known, but it may represent a self-propagating wave of mitochondria undergoing mPTP opening, with calcium and/or ROS released from one mitochondria on mPTP opening, initiating mPTP opening in the adjacent mitochondria and so on,([647;891]) until the whole cell has undergone global mitochondrial depolarisation. Several other studies have described mPTP-induced mitochondrial 'waves' of depolarisation, with the suggestion that they contribute to cellular Ca^{2+}-signalling([647;839]) or propagation of the apoptotic signal.([842])

In contrast, a recent study by O'Rourke's group documented oscillatory waves of mitochondrial membrane depolarisation and repolarisation, in response to the laser-induced local release of ROS in a small volume of the cell.([862]) They demonstrated that the oscillatory 'waves' of mitochondrial depolarisation/repolarisation were mediated by ROS-induced ROS release from the mitochondrial electron transport chain, and that they were insensitive to CsA (and were not associated with mitochondrial calcein efflux), suggesting that they occurred independent of the mPTP. However, they were blocked by the inner membrane anion channel (IMAC) inhibitor, DIDS (4'-chlorodiazepam), and a mitochondrial benzodiazepine receptor antagonist, which interestingly has been suggested to be a component of the mPTP.([719]) However, the investigators postulated that ROS (superoxide anion) exited via the IMAC and propagated the oscillatory mitochondrial waves of depolarisation/repolarisation.([862]) Interestingly, Vanden Hoek and colleagues when investigating the role of ROS as a trigger/mediator of hypoxic preconditioning, observed that the mitochondrial release of ROS was inhibited by the IMAC inhibitor, DIDS, suggesting that the IMAC was acting as an exit channel for mitochondrial generated ROS.([49])

The model used by Marban's group([862]) differs from the one in our study in that we subjected the whole cell to a much greater quantity of oxidative stress in order to induce mPTP opening and rigour contracture, and the 'wave' of mitochondrial depolarisation we observed

occurred as a single non-oscillatory wave that propagated from one end of the cell to the other, until the whole cell had undergone mitochondrial membrane depolarisation. As such the 'wave' of mitochondrial depolarisation, we observed constituted part of the process of mPTP opening.

5.7.6.5 The mechanism by which preconditioning inhibits mPTP opening

The mechanism by which preconditioning results in the inhibition of mPTP opening is unclear but it is probably due to a direct effect of preconditioning on mitochondrial function, such that preconditioned-mitochondria are rendered more resistant to the insult of ischaemia-reperfusion injury (discussed in section 5.10). The mechanism by which diazoxide inhibits the mPTP opening induced by oxidative stress is investigated in section 5.9.

5.8 Aim (6):

Determine whether opening the mitochondrial K_{ATP} channel at the time of reperfusion protects against lethal reperfusion injury by inhibiting mPTP opening

Pharmacological opening of the mitochondrial K_{ATP} channel at the time of reperfusion, following an episode of lethal ischaemia has been shown in the in vivo canine([268;269]) and swine([636]) models of ischaemia-reperfusion injury to be cardio-protective, though not in the in vivo rabbit model.([256;270;271]) In addition, Toyoda et al observed that 5-HD (the mitochondrial K_{ATP} channel blocker) administered at the time of reperfusion increased infarct size in IPC-treated hearts, suggesting that the mitochondrial K_{ATP} channel must be open during the reperfusion phase for preconditioning-induced protection.([169]) Using chick neonatal myocytes, Vanden Hoek and colleagues observed that hypoxic preconditioning reduced the production of ROS at the time of reoxygenation and that this effect could be blocked by 5-HD and mimicked by the K_{ATP} channel opener, pinacidil, suggesting that the opening of the mitochondrial K_{ATP} channel at the time of reperfusion may protect against lethal reperfusion injury by attenuating oxidative stress.([79])

Other studies however, have failed to show cardio-protection with the pharmacological activation of the mitochondrial K_{ATP} channel using either diazoxide([256]) or nicorandil.([270;271])

Having demonstrated in sections 5.4 and 5.5 that opening of the mK_{ATP} channel as part of a preconditioning protocol may protect the heart by inhibiting mPTP opening, we hypothesised that opening of the mK_{ATP} channel by administering diazoxide for the first 15 minutes of reperfusion protects the Langendorff-perfused rat heart by inhibiting mPTP opening.

5.8.1 Materials

Diazoxide (Sigma Chemicals, Poole, Dorset) was dissolved in dimethyl sulphoxide (DMSO) and added to the Krebs-Henseleit buffer such that the final DMSO concentration was less than 0.01%. All other reagents were of standard analytical grade.

5.8.2 Isolated Langendorff-Perfused Rat Heart Model

Hearts were excised from male Sprague-Dawley rats and mounted on a Langendorff-perfusion apparatus and subjected to 35 minutes regional ischaemia followed by 120 minutes of reperfusion (see section 3.2.1). At the end of the reperfusion period, the infarct-risk volume ratio was determined using tetrazolium (TTC) staining (see section 3.2.2).

5.8.3 Experimental Protocols for Infarct Studies

The hearts were randomly assigned to one of the following treatment groups:

(1) ***Control hearts*** were perfused with 0.01% DMSO (n=6) or Krebs-Henseleit buffer alone (n=6) at reperfusion for 15 minutes;

(2) ***Dzx-treatment***: hearts (n=6) were perfused with diazoxide (30 μmol/l) for the first 15 minutes of reperfusion. This concentration of diazoxide has been demonstrated to cardio-protect the isolated perfused rat heart.([104])

5.8.4 Results

5.8.4.1 Exclusions

We used 14 male Sprague-Dawley rat hearts for the infarct size experiments of which 0 were excluded (see section 3.2.2 for exclusion criteria).

5.8.4.2 Animal and Haemodynamic Data

Animal and heart weights and risk zone volumes were similar in the experimental groups (see table 5.10). Baseline data relating to cardiac function (assessed by the rate-pressure-product, RPP) and coronary flow before the lethal ischaemic period, were similar in the experimental groups (see tables 5.11, 5.12). During regional ischaemia, coronary flow and RPP decreased to a similar extent in both groups.

Table 5.10 Characteristics of Animals in Treatment Groups

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
1. Control	6	445±8.7	1.83±0.06	0.533±0.041
2. Diazoxide at reperfusion	8	403±18.1	2.38±0.13	0.517±0.016

Values are mean±SEM. *P<0.001 compared with control.

Table 5.11 Rate Pressure Product (x 10^3mmHg/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	23.9±3.9	27.4±2.4	17.8±2.3	17.5±5.6	28.1±2.4	13.1±0.1
2. Diazoxide at reperfusion	22.4±1.6	24.4±2.0	13.8±2.6	12.5±1.3	14.4±1.5	13.1±1.6

Values are mean±SEM. *P<0.005 compared with control.

Table 5.12 Coronary Flow Rate (ml/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	16.0±1.0	16.0±0.6	7.8±0.9	7.0±0.6	12.5±3.5	7.8±1.4
2. Diazoxide at reperfusion	19.7±1.3	19.7±1.3	8.7±0.4	8.5±0.5	11.2±0.7	6.3±0.3

Values are mean±SEM. *P<0.005 compared with control.

5.8.4.3 Infarct Size Data

Infarct size is represented as the percentage of tetrazolium-negative tissue in the ischaemic risk zone. Pharmacologically opening the mK_{ATP} channel by giving diazoxide for the first 15 minutes of reperfusion reduced infarct size from 44.8±3.2% in control hearts to 30.4±4.5% ($P<0.001$).

5.8.5 Discussion

In this section of the study, we demonstrated that the presence of diazoxide (a putative mitochondrial K_{ATP} channel) for the first 15 minutes of reperfusion protected the heart against lethal reperfusion injury, as evidenced by a reduction in infarct size. Our findings are in close agreement with studies in the open chest dog([268;269]) and swine,([636]) demonstrating a cardio-protective effect from activating the mitochondrial K_{ATP} channel at the time of reperfusion. Further studies are required to confirm the involvement of the mitochondrial K_{ATP} channel, and to determine whether diazoxide is protecting via the inhibition of the mPTP opening that occurs at the time of reperfusion. Diazoxide has been demonstrated to decrease oxidative stress at the time of reperfusion,([78]) which may be one possible mechanism by which diazoxide inhibits mPTP opening at the time of reperfusion. In addition, Garlid's group have proposed that mitochondrial K_{ATP} channel opening at the time of reperfusion would increase rapid energy conversion to phosphocreatine, with the effect that mitochondria do not produce a burst of ROS at reperfusion, such that opening of the mPTP does not occur.([110])

Having demonstrated from the previous sections that preconditioning may protect the heart by inhibiting mPTP opening at the time of reperfusion and that the mechanism linking preconditioning with mPTP inhibition may be the modulation of mitochondrial function, our next objective was to examine the effects of the preconditioning mimetic, diazoxide, on mitochondrial function.

5.9 Aim (7):

Determine the effect of preconditioning on mitochondrial redox potential and mitochondrial membrane potential.

Studies have implicated the modulation of mitochondrial function as an important mechanism in preconditioning-induced protection. IPC or preconditioning via the opening of the mitochondrial K_{ATP} channel have been demonstrated to confer beneficial effects on mitochondrial function which render the mitochondria and therefore the cell more resistant to the insult of ischaemia-reperfusion injury. These beneficial effects on mitochondrial function may also protect the heart against ischaemia-reperfusion injury by inhibiting the mPTP opening that occurs at the time of reperfusion. The mitochondrial effects of preconditioning include: (1) the attenuation of mitochondrial Ca^{2+} loading;([52;239;257;258;261;262]) (2) rendering the mitochondria more resistant to mitochondrial Ca^{2+} loading;([263-265]) (3) maintaining mitochondrial energy production;([159;234;267]) (3) reducing the production of oxidative stress at the time of reperfusion;([77-79;278]) (4) mitochondrial uncoupling.([150;236;237])

However, several aspects concerning the effects of IPC or mitochondrial K_{ATP} channel opening on mitochondrial function are shrouded with controversy. For example, the issue of whether the opening of the mitochondrial K_{ATP} channel actually induces mitochondrial membrane depolarisation is currently under debate. Several studies have demonstrated mitochondrial membrane depolarisation in response to diazoxide,([52;239;257;258;846]) but some studies have failed to observe this effect of diazoxide([109;159;160]) and attribute it to a non-specific effect of the drug due to the use of high concentrations([160;260]) or else the fact that the cells or mitochondrial under examination were de-energised.([110;846])

A further controversial area is the use by Marban's group, of flavoprotein oxidation, that occurs as a consequence of mitochondrial membrane depolarisation, to indicate opening of the mitochondrial K_{ATP} channel.([108;143]) Other studies have failed to demonstrate either mitochondrial membrane depolarisation([109;159;160]) or flavoprotein oxidation([109]) with mitochondrial K_{ATP} channel activation.

On this background, we examined the effect of diazoxide on mitochondrial redox potential and mitochondrial membrane potential.

5.9.1 Materials

Diazoxide (Sigma Chemicals, Poole, Dorset) was dissolved in dimethylsulphoxide (DMSO), giving a final concentration of <0.1% DMSO. Tetramethylrhodamine methyl ester (TMRM, Molecular Probes Europe BV, Leiden, The Netherlands) was dissolved in DMSO. All other agents were of standard analytical grade and quality.

5.9.2 Myocyte studies

Adult rat myocytes were isolated from male Sprague-Dawley rats according to the method in described in section 3.5. Isolated myocytes were seeded onto 25-mm round cover-slips according to the method in described in section 3.6.

5.9.2.1 Effect of Preconditioning on Mitochondrial Redox Potential

The effect of the preconditioning mimetic, diazoxide on the mitochondrial redox potential (assessed by measuring FAD^+ or NADH autofluorescence, using confocal microscopy) in quiescent cells was determined using two treatment protocols:

(a) ***Acute effect***: micropipetting diazoxide (30 μmol/l) onto the myocyte over 5 minutes. This concentration of diazoxide has been shown to as a preconditioning mimetic;([104]) and

(b) ***Sub-acute effect***: cells were either incubated with diazoxide (30 μmol/l) or 0.1% DMSO for 30 minutes at 37°C.

Mitochondrial NADH and FAD^+ fluorescence were measured from 20 cells in each treatment group.

Next the effect of the preconditioning mimetic, diazoxide on the mitochondrial redox potential (assessed by measuring NADH autofluorescence, using confocal microscopy-see section 3.7) in cells subjected to the TMRM oxidative stress protocol described in section 5.6.2 was determined. After loading with TMRM in the restoration buffer, cells were randomly assigned to one of the following treatment groups. Cells were incubated for 20 minutes with the drug(s) and were then washed with restoration buffer and subjected to the TMRM-oxidative stress protocol at 37°C.

1. ***Control***-incubation with restoration buffer alone (n=18), or 0.1% DMSO vehicle control (n=6);
2. ***Diazoxide*** (30 μmol/l, n=6). This concentration of diazoxide has been shown to act as a preconditioning mimetic;([104])

NADH auto-fluorescence was elicited using the 351 laser line of the Zeiss 510 CLSM after careful pinhole alignment of the microscope so that images could be acquired with minimal illumination intensities for prolonged period with no apparent adverse effects and no photo-bleaching. The NADH fluorescence signal was collected using a band-pass filter between 375 and 488 nm. A configuration using multi-tracking (switching between configurations from frame to frame) to allow simultaneous measurements of flavoprotein and NADH fluorescence. The flavoprotein (FAD) signals were excited using the 458 line of an argon laser and were measured at >505 nm. Flavo-protein signals increase with oxidation and decrease with reduction, while NADH signals show opposite changes. The signals were 'calibrated' in terms of maximally oxidised and maximally reduced by using cyanide (2 mmol/l) to promote maximal reduction and FCCP (1 mmol/l) for maximal oxidation.

5.9.2.2 Effect of Preconditioning on Mitochondrial Membrane Potential

The effect of the preconditioning mimetic, diazoxide on the mitochondrial membrane potential (assessed by TMRM fluorescence, using confocal microscopy-see section 3.7) in quiescent cells were determined using two treatment protocols:

(a) ***Acute effect***: Cells were incubated with 3 µmol/l of TMRM for 30 minutes, to allow the TMRM in the bathing solution to equilibrate with the mitochondria. Diazoxide (30 µmol/l) was then micropipetted onto the myocyte over 5 minutes. This concentration of diazoxide has been shown to act as a preconditioning mimetic;([104]) and

(b) ***Sub-acute effect***: Cells were incubated with 50 nmol/l of TMRM for 30 minutes, to allow the TMRM in the bathing solution to equilibrate with the mitochondria. Cells were either incubated with diazoxide (30 µmol/l) or 0.1% DMSO for 30 minutes at 37°C.

5.9.3 Results

5.9.3.1 Effect of Preconditioning on Mitochondrial Redox Potential

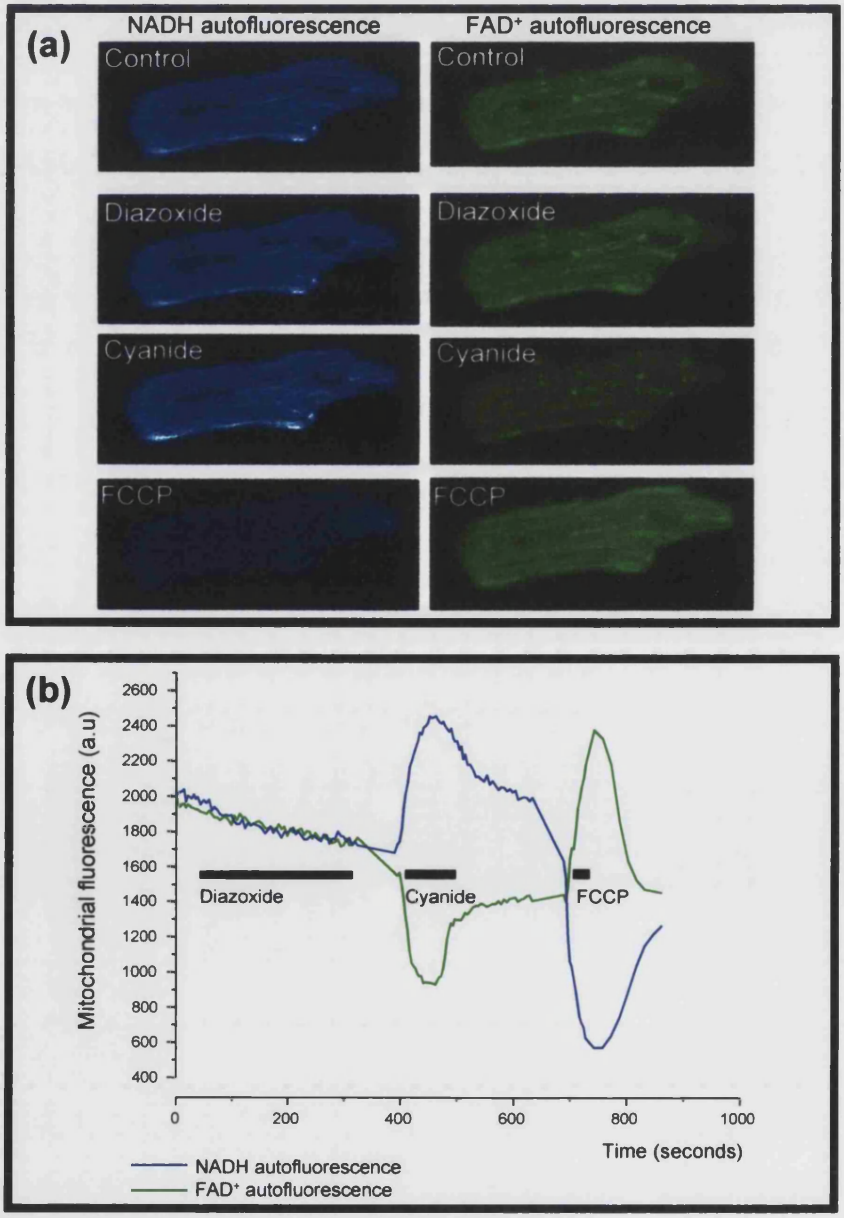
5.9.3.1.a The Acute Effect of Diazoxide on Quiescent Cells

Micro-pipetting diazoxide over 5 minutes onto a myocyte had no acute effect on either FAD^+ or NADH autofluorescence (figure 5.16a,b). Cyanide as expected increased NADH fluorescence and decreased FAD^+ fluorescence, whereas FCCP reduced NADH fluorescence and increased FAD^+ fluorescence (figure 5.16a,b).

5.9.3.1.b The Sub-Acute Effect of Diazoxide on Quiescent Cells

There was no change in NADH autofluorescence in cells incubated with diazoxide (30 µmol/l) for 30 minutes (148.3±8.1 arbitrary units [a.u] after incubating with DMSO 0.1% for 30 minutes compared to 164.1±7.7 a.u after incubating with diazoxide for 30 minutes; N=20; P=NS). However, there was a small but significant increase in FAD^+ autofluorescence in cells incubated with diazoxide (30 µmol/l) for 30 minutes (178.8±3.5 arbitrary units [a.u] after incubating with DMSO 0.1% for 30 minutes compared to 197.9±4.7 a.u after incubating with diazoxide for 30 minutes; N=20; P<0.05).

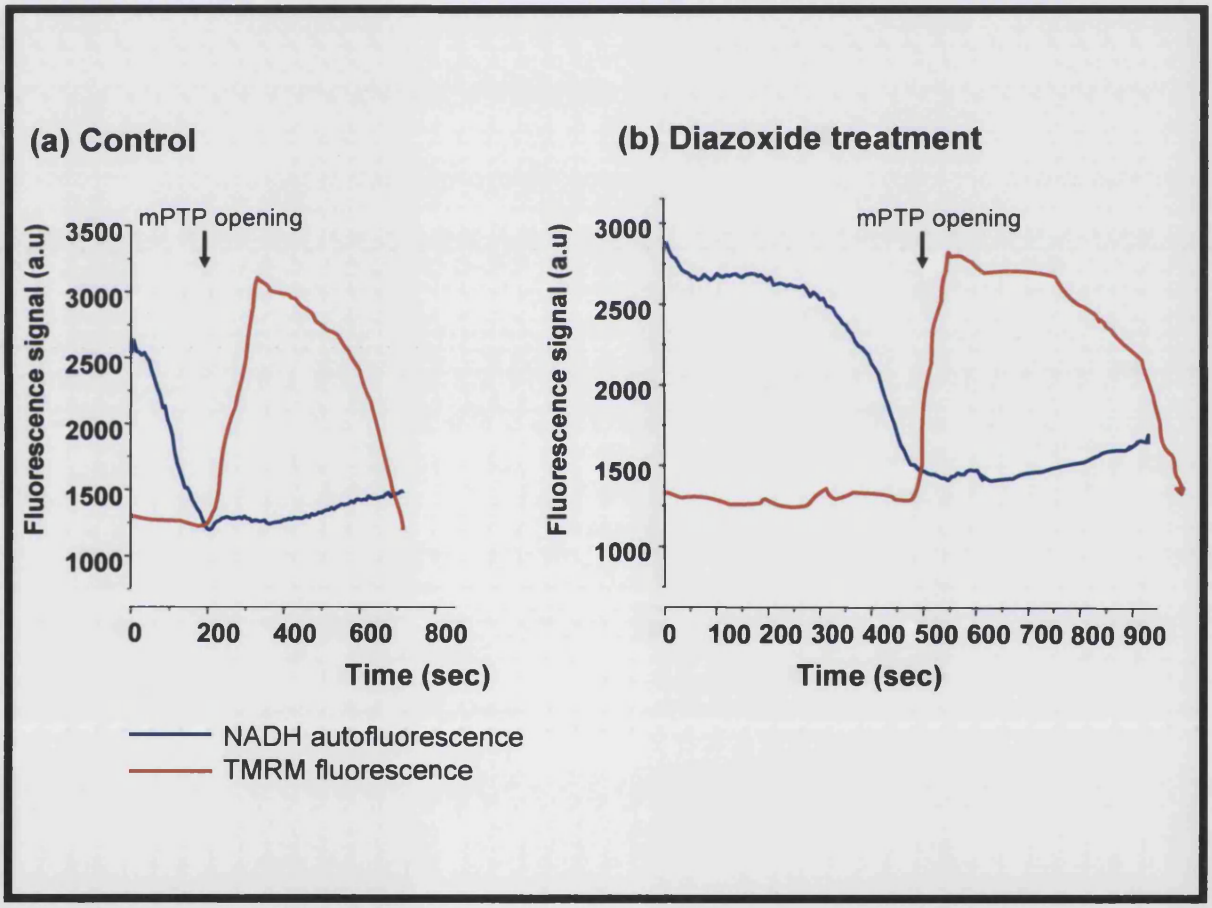
Figure 5.16: The Acute Effect of Diazoxide on Mitochondrial Redox Potential: Diazoxide micropipetted onto myocytes (over 5 minutes) followed by cyanide (2 mmol/l) and FCCP (1 mmol/l) on NADH and FAD^+ fluorescence in a representative myocyte (a) Confocal images; (b) Graph showing changes in NADH and FAD^+ fluorescence signal over time.



5.9.3.1.c The Effect of Diazoxide on Cells Subjected to Oxidative Stress

Diazoxide treatment of cells prior to the TMRM-induced oxidative stress protocol not only delayed the time taken to induce mPTP opening and rigour contracture, but also appeared to attenuate the drop in NADH auto-fluorescence associated with the oxidative stress (figure 5.17 a,b).

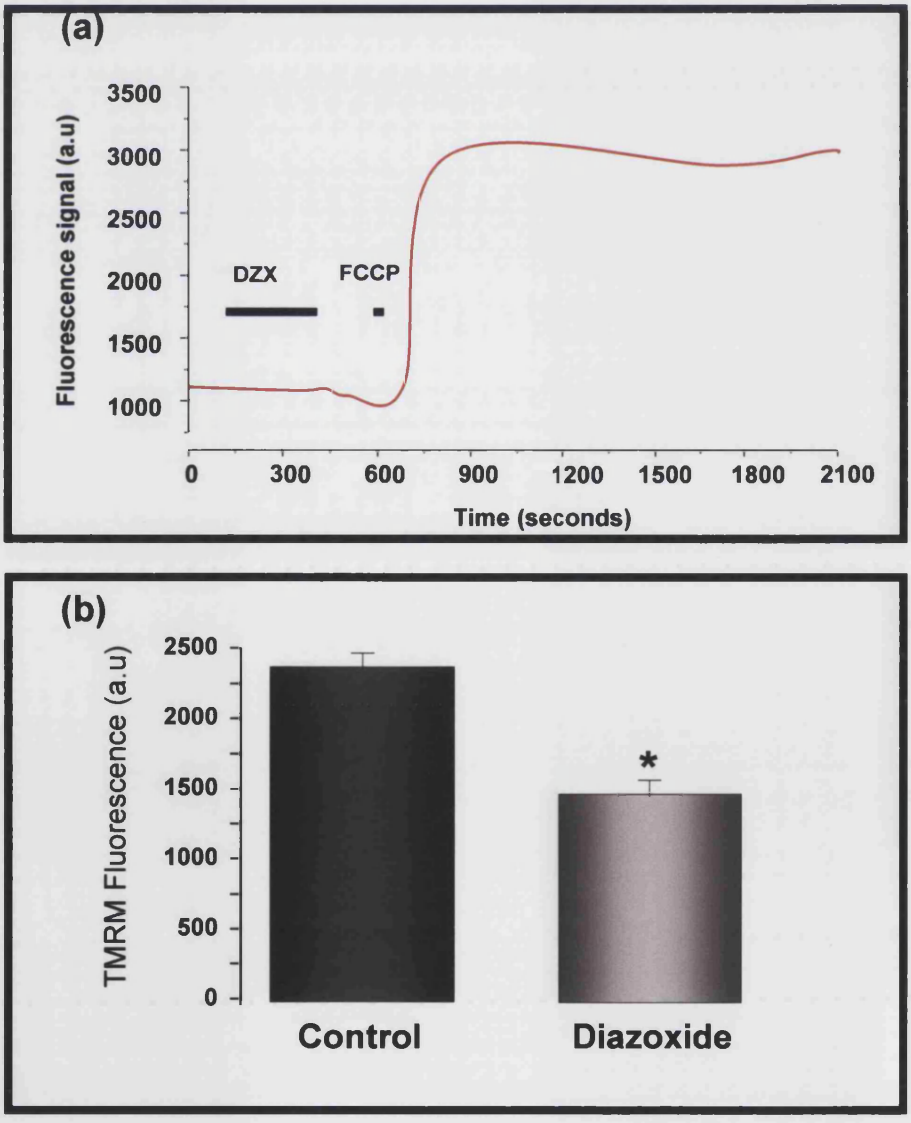
Figure 5.17 a,b: The Effect of Diazoxide on NADH Autofluorescence in Cells Subjected to Oxidative Stress. (a) In the control cell there was a sharp decline in NADH autofluorescence with oxidative stress, which slowly rises after mPTP opening has occurred. (b) Diazoxide treatment of cells delays the time taken to induce mPTP opening and rigour contracture, and also appeared to attenuate the drop in NADH auto-fluorescence associated with the oxidative stress. However, after mPTP opening, the NADH fluorescence slowly rises.



5.9.3.2 Effect of Preconditioning on Mitochondrial Membrane Potential

Acute administration of diazoxide micro-pipetted onto the myocyte had no immediate effect on mitochondrial membrane potential, as assessed by TMRM fluorescence (N=10, figure 5.18a), even though complete mitochondrial depolarisation occurred with FCCP (1 mmol/l). However, after incubation with diazoxide for 30 minutes, there was a significant reduction in TMRM fluorescence (from 2149.4±100.7 in control to 1498.0±74.5 arbitrary units of fluorescence; N=20; P<0.001; figure 5.18b), suggesting significant mitochondrial membrane potential depolarisation.

Figure 5.18 a,b: The Effect of Diazoxide on Mitochondrial Membrane Potential. (a) Diazoxide had no acute effect on mitochondrial membrane potential when given over 5 minutes, whereas the uncoupler FCCP induces a complete collapse in mitochondrial membrane potential, as evidenced by an increase in TMRM signal signifying dequenching. (b) However, when the cells were incubated with diazoxide for 30 minutes there was a significant reduction in TMRM fluorescence, indicating mitochondrial membrane depolarisation.*P<0.001 compared to control.



5.9.4 Discussion

5.9.4.1 The Effect of Diazoxide on Mitochondrial Redox Potential in Quiescent Cells

We demonstrated in this part of the study that the acute administration (over 5 minutes) of diazoxide (at a concentration associated with cardio-protection)([104]) had no effect on mitochondrial redox potential, as measured by NADH and FAD^+ autofluorescence. However, when diazoxide was administered sub-acutely (incubation for 30 minutes) there was a small but significant increase in FAD^+ autofluorescence, though no change in NADH autofluorescence. Marban's group have demonstrated a rapid (within 5 minutes) flavoprotein oxidation with diazoxide (at comparable concentrations), and they have used this effect to indicate mitochondrial K_{ATP} channel opening.([108;143;889]) However, we only observed flavoprotein oxidation after incubating the cells with diazoxide for 30 minutes. In contrast, Standen's group,([109]) have demonstrated no change in flavoprotein oxidation with a comparable concentration of diazoxide given over 15 minutes. The reason for the difference probably relates to the fact that the effect of diazoxide on mitochondrial flavoprotein oxidation may be only observed in de-energised myocytes, that had been kept overnight in substrate-free medium.([110])

5.9.4.2 The Effect of Diazoxide on Mitochondrial Redox Potential in Cells Subjected to Oxidative Stress

In order to investigate the effect of the preconditioning mimetic diazoxide on mPTP opening, we used a model in which mPTP opening in myocytes was induced by laser-induced oxidative stress (see section 5.7). We demonstrated that diazoxide pre-treatment prolonged the time taken to induce both mPTP opening and rigour contracture, suggesting that diazoxide protects the cell against oxidative stress by inhibiting mPTP opening. In this part of the study, we examined the effect of diazoxide on NADH autofluorescence in the setting of oxidative stress. In control cells, there was a rapid decline in NADH autofluorescence, due to oxidation of NADH, followed by the opening of the mPTP (indicated by mitochondrial membrane depolarisation). We found that diazoxide appeared to attenuate the drop in NADH auto-fluorescence associated with oxidative stress, when compared to control cells. The delay in the decline of the NADH autofluorescence in the presence of diazoxide appears to correlate with the delay in inducing mPTP opening. This finding suggests that diazoxide was preserving NADH autofluorescence via a possible free-radical scavenging effect. The oxidation of NADH is a critical determinant of

mPTP opening, and the reduction in NADH oxidation, may be a possible mechanism by which diazoxide inhibits mPTP opening. Interestingly, several studies have demonstrated that diazoxide can reduce the production of oxidative stress.([77-79;120;121;278])

5.9.4.3 The Effect of Diazoxide on Mitochondrial Membrane Potential

A possible mechanism for diazoxide-induced protection against ischaemia-reperfusion injury, is that it reduces the mitochondrial membrane potential that drives the influx of Ca^{2+} into the mitochondria, thereby attenuating Ca^{2+} mitochondrial loading during ischaemia-reperfusion.([52;108;239;257;258;846]) However, whether diazoxide induces mitochondrial membrane depolarisation is in dispute.([109;159;160;259])

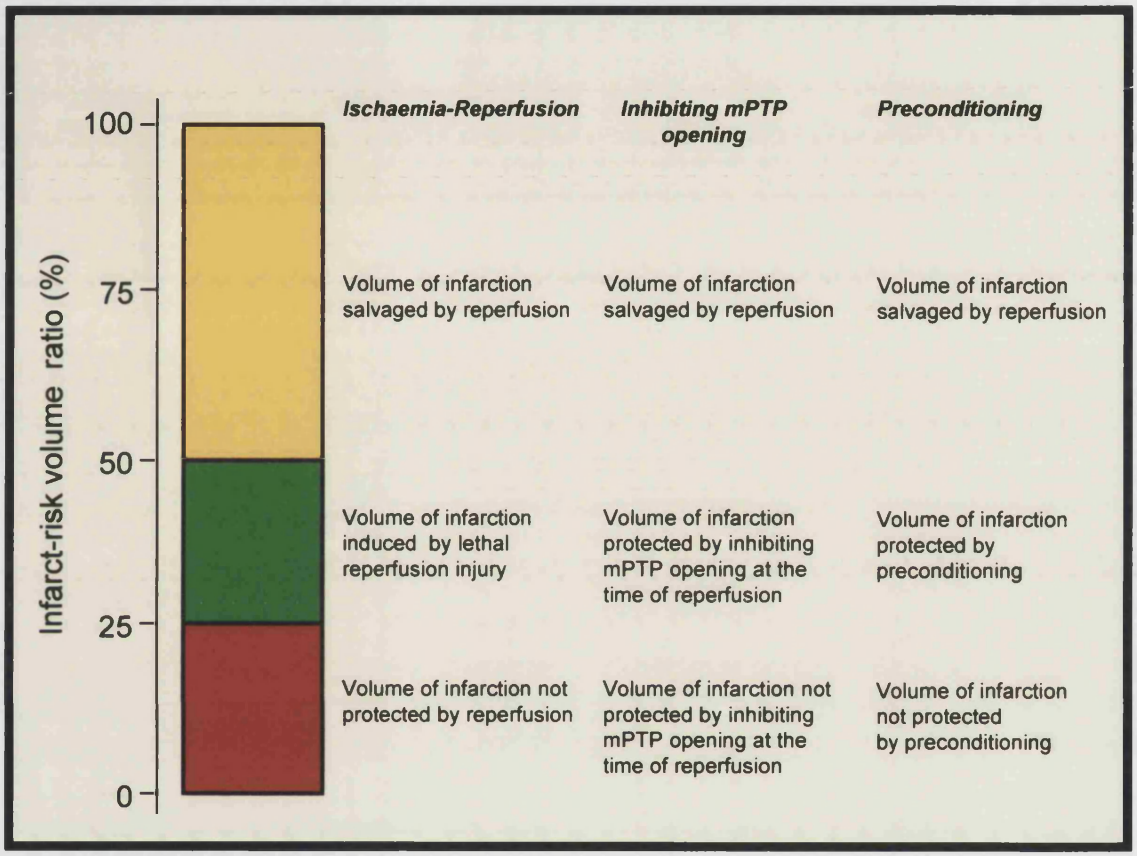
Interestingly, we demonstrated that acutely (over 5 minutes), diazoxide had no effect on mitochondrial membrane potential. However, after 30 minutes incubation with diazoxide we did observe a significant reduction in mitochondrial membrane potential. The reason why we observed mitochondrial membrane depolarisation after a prolonged incubation is not clear, but it may be related to the inhibitory effect of diazoxide on mitochondrial respiration. Diazoxide has been demonstrated to inhibit succinate dehydrogenase, which forms part of the FADH-linked electron transport carrier,([107;121;135;136;148;149]) the effect of which after a prolonged period of time, would be expected to depolarise the mitochondrial membrane potential. The question remains as to whether the observed depolarisation in mitochondrial membrane potential we observed in our model is of patho-physiological importance with respect to mitochondrial Ca^{2+} loading during ischaemia-reperfusion, as suggested by several studies.([52;108;239;257;258;846]).

5.10 Summary and Discussion

In this chapter, we have demonstrated that both ischaemic preconditioning (IPC) and pharmacological preconditioning using diazoxide, nicorandil and CCPA protect the heart against lethal reperfusion injury by inhibiting mPTP opening at the time of reperfusion. In sections 5.4 and 5.5, we demonstrated that pharmacologically opening the mPTP during the first 15 minutes of reperfusion, abrogated the protective effect associated with IPC, pharmacological preconditioning using diazoxide, nicorandil and CCPA, as well as CCPA-induced delayed preconditioning. In sections 5.6 and 5.7, we demonstrated that preconditioning protected by inhibiting the mPTP opening induced by mitochondrial Ca^{2+} loading and oxidative stress, two major determinants of mPTP opening at the time of reperfusion.

An important interpretation of the findings from this study is that preconditioning protects by modifying events which occur during the first few minutes of reperfusion, a theme which is explored further in the next chapter. Specifically, preconditioning appears to protect the heart against lethal reperfusion injury by rendering mitochondria more resistant to the crucial first few minutes of reperfusion, when the prevailing conditions predispose to mPTP opening and subsequent cell death. The scheme portrayed in figure 5.19 demonstrates in terms of infarct size the individual effects of lethal reperfusion injury, inhibition of mPTP opening at the time of reperfusion, and preconditioning. Importantly, the interpretation of the results from this chapter, could be that the volume of infarction reduced by preconditioning may be due to the inhibition of mPTP opening, which in turn protects the heart against lethal reperfusion injury.

Figure 5.19: Hypothetical Scheme Relating Lethal Reperfusion Injury, Inhibition of mPTP Opening and Preconditioning in Terms of Protection From Infarction. The protection against lethal reperfusion injury obtained from inhibiting mPTP opening at the time of reperfusion, may be the mechanism by which preconditioning protects the heart against ischaemia-reperfusion injury.



5.10.1 Evidence From Other Studies Supporting a Role for the mPTP in Preconditioning

Several studies have since been published confirming the findings in our study that the inhibition of mPTP opening is critical to preconditioning-induced protection (see table 5.13).

Table 5.13: Table Listing Studies Which Have Implicated the mPTP in Preconditioning

Group	Model	PC stimulus	Note
Ashraf 2001([251])	Rat myocytes	Calcium	Atr abrogates protection
Weiss 2002([846])	Rabbit mitochondria	Diazoxide	Inhibition of mPTP
Dos Santos 2002([159])	Rat mitochondria	Diazoxide	Reduced mitochondrial Cytochrome C release
Baines 2003([881])	Mice mitochondria	IPC	Inhibition of mPTP
Halestrap 2003([803])	Rat heart	IPC	Inhibition of mPTP
Lemasters 2003([892])	Rat mitochondria	Heat shock	Inhibition of mPTP
Akao 2003([859])	Myocytes	mK_{ATP}	Delayed mPTP opening
Rajesh 2003([882])	Rat mitochondria	SWOP	Inhibition of mPTP

The first study by Ashraf's group,([251]) demonstrated that opening the mPTP using atractyloside, in neonatal rat myocytes subjected to anoxia-reoxygenation injury, reversed the protective effects of calcium-preconditioning. However, in this study, a direct inhibitory effect of calcium-preconditioning on mPTP opening was not demonstrated.([251])

A subsequent study by Weiss' group([846]) demonstrated, using isolated rabbit mitochondria, that both diazoxide and a PKC agonist could inhibit calcium-induced mPTP opening (detected by the mitochondrial efflux of calcein), which confirms our findings from section 5.6, in which we demonstrated diazoxide-induced inhibition of calcium-induced mPTP opening.

Garlid's group([159]) demonstrated that diazoxide preserved the permeability of the outer mitochondrial membrane during ischaemia-reperfusion such that cytochrome C release was prevented. One potential mechanism for cytochrome C release is mPTP opening, and therefore this study was indirectly demonstrating that diazoxide can induce mPTP opening. In the same

vein other studies reporting reduced mitochondrial cytochrome C release may have indirectly implicated the mPTP in preconditioning.([251;274])

This was followed by a study from Baines and colleagues([881]) demonstrating the inhibition of m PTP opening (detected by the mitochondrial swelling) in preconditioned mice hearts. Importantly, this study addressed the issue of how preconditioning mediates the inhibition of mPTP opening (see later). Halestrap's group([803]) demonstrated in the isolated perfused rat heart that IPC result in the inhibition of mPTP opening (detected by the mitochondrial 2-deoxyglucose entrapment technique) at the time of reperfusion, though the mitochondria isolated from IPC-treated hearts were found to be more sensitive to calcium-induced mPTP opening than control hearts (see section 5.10.2). Akao and colleagues([859]) demonstrated using neonatal rat myocytes, that diazoxide, nicorandil and pinacidil delays the onset of mPTP opening (detected as mitochondrial membrane depolarisation) induced by administered hydrogen peroxide.

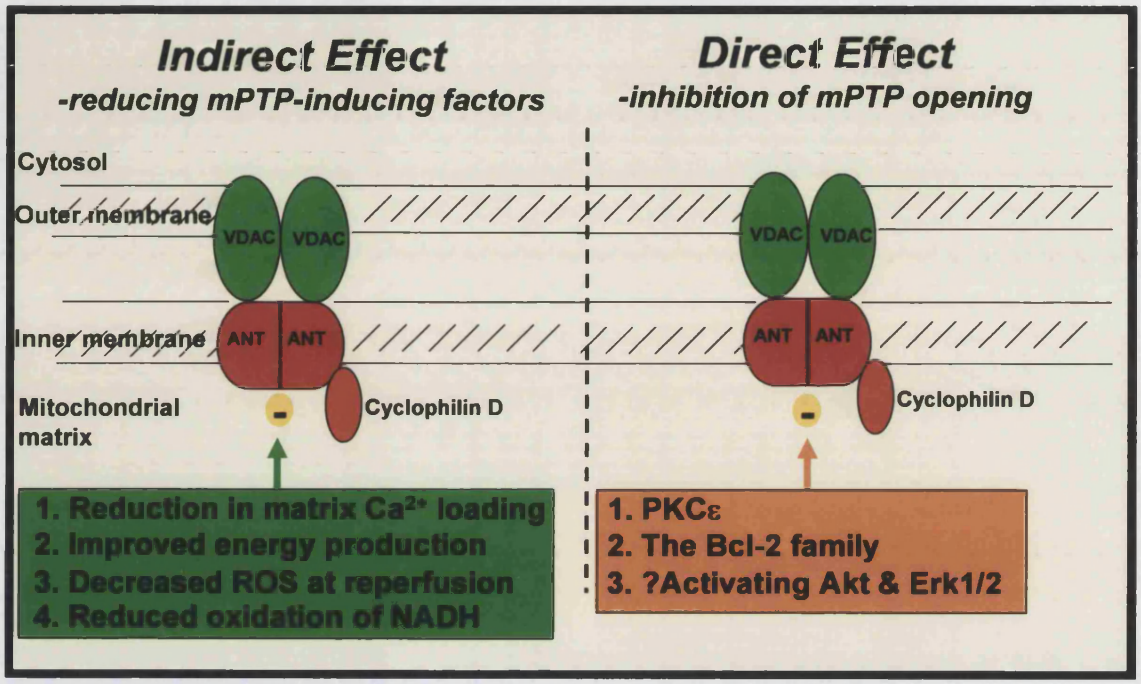
The second window of protection has been investigated by Rajesh and colleagues([882]) who demonstrated that the protective effects present 24 hours after an IPC protocol, were abrogated if the mPTP was opened just prior to the index ischaemic period. However, in this study, the direct effect of the SWOP on mPTP opening was not examined. Finally, Lemaster's group([892]) examined the role of the mPTP in heat shock, which is known to induce a SWOP.([44]) They demonstrated that mitochondria isolated from rat livers that had been subjected to heat shock were less sensitive to calcium-induced mPTP opening. ([892])

Therefore, the role of the mPTP in myocardial preconditioning is becoming more apparent, though the question still remains as to how preconditioning inhibits mPTP opening.

5.10.2 Mechanisms by Which Preconditioning Inhibits mPTP Opening

The most obvious mechanism by which preconditioning leads to inhibition of mPTP opening at the time of reperfusion is an indirect effect (see figure 5.20), in which preconditioning confers a beneficial effect on mitochondrial function such that at the time of reperfusion, the mitochondria are more resistant to mPTP opening.

Figure 5.20: Hypothetical Scheme Depicting the Potential Mechanisms by Which Preconditioning may Inhibit mPTP Opening. Preconditioning-mediated inhibition of mPTP opening could be a mediated by an indirect effect whereby preconditioning reduces mPTP inducing factors such that mPTP opening at the time of reperfusion is inhibited. Alternatively the preconditioning-mediated inhibition of mPTP opening could be a direct effect whereby a component of preconditioning inhibits mPTP opening directly (see text for details).



The fact that preconditioning can modify the same factors that predispose to mPTP opening during the first few minutes of reperfusion supports this mechanism. In this regard, the major determinants of mPTP opening at the time of reperfusion are: (1) the mitochondrial Ca^{2+} load, which is attenuated in the setting of preconditioning.([52;239;257;258;261;262]) In addition, preconditioning renders the mitochondria more resistant to mitochondrial Ca^{2+} loading;([263-265]) (2) ATP depletion. Preconditioning has been demonstrated to improve mitochondrial energy production;([159;234;267]) (3) Oxidative stress which induces mPTP opening via cross-linking of thiol group and the oxidation of NADH. Preconditioning has been demonstrated to attenuate the production of oxidative stress at the time of reperfusion.([77-79;278]) We have also demonstrated that diazoxide appears to attenuate the oxidation of NADH in the setting of oxidative-stress-induced mPTP opening (see section 5.9).

In apparent support for an indirect effect of preconditioning on mPTP opening, Halestrap's group([803]) demonstrated that mitochondria isolated from IPC-treated hearts that had demonstrated inhibition of mPTP opening in situ, were paradoxically more sensitive to mPTP

opening when isolated, suggesting that the factor (s) mediating the inhibition of mPTP opening were lost in the isolation procedure.([803])

However, an alternative mechanism through which preconditioning inhibits mPTP opening, may be a direct effect on the mPTP (see figure 5.20). In this scenario, a component of preconditioning appears to target and inhibit mPTP opening directly. There are three possible candidates in this scenario:

5.10.2.1 Protein Kinase C

Baines and colleagues([881]) have demonstrated that PKC-ε (which has been implicated as a mediator of preconditioning-induced protection) co-immuno-precipitated with components of the mPTP, including the VDAC and ANT. Using isolated mitochondria, they demonstrated that exogenous PKC-ε inhibited calcium-induced mPTP opening in isolated mouse mitochondria. This suggests that a known component of preconditioning, PKC, can interact with and inhibit mPTP opening. Whether, this actually happens in the setting of ischaemia-reperfusion is unknown and remains to be tested. Several other components have been demonstrated to associate with components of the mPTP and regulate the function of the VDAC-ANT complex, including the mitochondrial benzodiazepine receptor,([719]) glycerol kinase,([893]) hexokinase([714]) and creatine kinase.([714;717])

Interestingly, the PKC-δ isoform, which has been recently implicated as a mediator of lethal reperfusion injury in an open chest pig model,([184;185]) may act by inducing mPTP opening. Therefore, preconditioning may inhibit mPTP opening by down-regulating PKC-δ, suggesting that the ratio between the PKC-ε/PKC-δ, may determine the effect of preconditioning on mPTP opening. Furthermore, Mochly-Rosen's group have demonstrated that the protection in the isolated perfused rat heart associated with the inhibition of PKC-δ and the activation of PKC-ε was additive.([185]) Another pairing that is modified by preconditioning and may influence mPTP opening in the setting of ischaemia-reperfusion injury, is that between the pro- and anti-apoptotic members of the Bcl-2 family.

5.10.2.2 The Bcl-2 Family

The ratio between certain pro-apoptotic members (Bax/Bak/Bad/BNIP3) and anti-apoptotic members (Bcl-2/BclX_L) of the Bcl-2 family have been demonstrated to be a major determinant of mPTP opening and apoptotic cell death (see section 1.5.4.2.a).([755-758]) Importantly, the anti-

apoptotic members, have been demonstrated to inhibit mPTP opening, and the pro-apoptotic members Bax, Bak and BNIP3 have been demonstrated by some investigators to induce mPTP opening.([755-762;762;763]) Interestingly, preconditioning has been demonstrated to protect the heart by up-regulating the anti-apoptotic proteins and down-regulating the pro-apoptotic proteins. ([243;244;882;894]) Therefore preconditioning may inhibit mPTP opening by modulating the ratio of pro- and anti-apoptotic proteins of the Bcl-2 family.

5.10.2.3 The Pro-Survival Kinases

The pro-survival kinases PI3K-Akt and MEK1/2-Erk1/2 as a potential link between preconditioning and the inhibition of mPTP opening at the time of reperfusion is examined in the next chapter.

5.11 Conclusion

In this section of the study we have demonstrated that myocardial preconditioning protects the heart against ischaemia-reperfusion injury by inhibiting the mPTP opening that occurs in the first few minutes of reperfusion. The findings suggest that preconditioning protects the heart by modifying crucial events such as mPTP opening, which take place at reperfusion. This may be because preconditioned-mitochondria are more resistant to ischaemia-reperfusion injury, such that at the time of reperfusion, mPTP opening is suppressed. This view is supported by the fact that many of the factors that induce mPTP opening at the time of reperfusion can be neutralised by preconditioning. An alternative explanation could be that a component of preconditioning such as PKC or the Bcl-2 family, inhibits mPTP opening directly. In this regard the next chapter investigates the role of the pro-survival kinase cascades, PI3K-Akt and MEK1/2-Erk1/2 as a potential component of preconditioning which mediates protection by inhibiting mPTP opening.

The data from the infarct studies and mitochondrial experiments presented in this section have been published.([873])

Chapter Six

THE ROLE OF THE PRO-SURVIVAL KINASES Akt AND Erk1/2 IN MYOCARDIAL PRECONDITIONING

6.1 Introduction

The findings from chapter 5 demonstrated that myocardial preconditioning protects the heart by inhibiting the mPTP opening which occurs at the time of reperfusion. However, the mechanism by which preconditioning inhibits mPTP opening is unclear although several mechanisms have been postulated (see section 5.10.2). At the end of the last chapter we speculated that a potential candidate in this scheme could be the pro-survival phosphatidylinositol 3-OH kinase (PI3K)-Akt, and possibly the mitogen activated protein kinase (MAPK), p42/p44 extra-cellular signal-regulated kinases (Erk1/2) cascades. Therefore, in this chapter we investigated the role of the PI3K-Akt and MEK1/2-Erk1/2 kinase cascades ***at the time of reperfusion*** in hearts that have undergone ischaemic preconditioning (IPC), to determine whether these kinases are required for IPC-induced protection and if they are, do they protect the heart by inhibiting mPTP opening.

The PI3K-Akt and MEK1/2-Erk1/2 kinase cascades are activated in the setting of ischaemia-reperfusion and mediate cellular protection by recruiting anti-apoptotic mechanisms outlined in section 1.4.3. Recent studies have suggested that the activation of either the PI3K-Akt,([80-82]) and possibly the Erk1/2 cascades,([82;194]) relays the ischemic and pharmacological preconditioning signal to downstream mediators of preconditioning such as PKC([81]) and reactive oxygen species (ROS).([80]) In this scenario, these kinase cascades are activated in response to an IPC stimulus during the preconditioning phase, prior to the lethal ischaemic episode. We were interested in the role of these kinases at the time of reperfusion, following the lethal ischaemic episode in the setting of IPC.

The roles of the PI3K-Akt and MEK1/2-Erk1/2 kinase cascades at the time of reperfusion in the context of mediating protection against lethal reperfusion injury have been investigated extensively by Yellon's group.([519]) They have previously shown that pharmacologically activating these kinases at the time of reperfusion, by administering growth factors (such as insulin,([530;895]) TGF-β1,([534]) CT-1,([525]) and urocortin([535;536])) or other agents (such as atorvastatin([522]) and bradykinin([524])) during the first few minutes of reperfusion, protects the heart by limiting both the apoptotic and necrotic components of cell death (see section 1.4.3.1), through the phosphorylation of BAD, p70S6K([530]) and eNOS.([524]) This suggests that activating these pro-survival kinase cascades at the time of reperfusion, which

together comprise the Reperfusion Injury Salvage Kinase (RISK)-Pathway, can protect the heart against lethal reperfusion injury (see section 1.4.3).

Whether these kinase cascades are activated at the time of reperfusion in response to an IPC stimulus, and whether their activation at this time contributes to IPC-induced protection is unknown. Therefore the initial aim of this part of the study was to determine whether IPC results in the activation of the PI3K-Akt and MEK1/2-Erk1/2 kinase cascades, at the time of reperfusion, following the lethal ischaemic insult, and whether the activation of these pro-survival kinases at reperfusion are essential for IPC-induced protection. The second part of the study was to determine whether these kinase cascades protect the heart by inhibiting mPTP opening.

6.2 Hypothesis

Myocardial preconditioning protects by inhibiting the prolonged (high-conductance) mPTP opening at the time of reperfusion, following the lethal ischaemic insult, via the activation of the pro-survival kinases Akt and Erk1/2.

The first part of the study (section 6.3) was to determine, using an isolated perfused rat heart, whether IPC results in the activation of the PI3K-Akt and MEK1/2-Erk1/2 kinase cascades at the time of reperfusion, following the lethal ischaemic insult, and if it did, to determine whether these kinases are essential for IPC-induced protection. The next part of the study (section 6.4) was to determine if these kinase cascades protect the heart by inhibiting mPTP opening.

6.3 Aim (1):

Determine whether myocardial preconditioning activates the pro-survival kinases Akt and Erk1/2 at the time of reperfusion, following the lethal ischaemic insult, and whether they are required for protection.

6.3.1 Materials

LY294002 (LY, Tocris, Bristol, UK), PD98059 (PD, Tocris, Bristol, UK), rapamycin (RAPA, Tocris, Bristol, UK) and chelerythrine (Chel, Sigma Chemicals, Poole, Dorset) were dissolved in dimethylsulphoxide (DMSO, Sigma Chemicals, Poole, Dorset) and added to the Krebs-Henseleit buffer such that the final DMSO concentration was less than 0.01%, which did not influence either infarct size or kinase phosphorylation. All other reagents were of standard analytical grade.

6.3.2 Isolated Langendorff-Perfused Rat Heart Model

Hearts were excised from male Sprague-Dawley rats and mounted on a Langendorff-perfusion apparatus and subjected to 35 minutes regional ischaemia followed by 120 minutes of reperfusion (see section 3.2.1). At the end of the reperfusion period, the infarct-risk volume ratio was determined using tetrazolium (TTC) staining (see section 3.2.2).

6.3.3 Experimental Protocols for Infarct Studies

The experiment protocols for the infarct studies are presented in Figure 6.1. The hearts were randomly assigned to one of the following treatment groups:

(1) ***Control hearts*** were given 0.01% DMSO (n=6) or Krebs-Henseleit buffer alone (n=6) for the first 15 minutes of reperfusion;

(2) ***IPC hearts*** (n=6) were treated with two-5 minute periods of global ischaemia/10 minute reperfusion periods prior to the lethal ischaemic insult;

(3) ***IPC + LY:*** IPC-treated hearts (n=6) were given LY 294002 (15 μmol/L, a specific PI3K inhibitor)([896]) for the first 15 minutes of reperfusion following the lethal ischaemic period. This concentration of LY 294002 has been shown to inhibit Akt phosphorylation in the isolated perfused rat heart;([82])

(4) ***IPC + PD:*** IPC-treated hearts (n=6) were given PD 98059 (10 μmol/L, a specific MEK1/2 inhibitor)([897]) for the first 15 minutes of reperfusion following the lethal ischaemic period. This

concentration of PD 98059 has been shown to inhibit Erk1/2 phosphorylation in the isolated perfused rat heart;([82])

(5) ***IPC + RAPA:*** IPC-treated hearts (n=6) were given rapamycin (0.5 nmol/L, a p70S6K inhibitor) for the first 15 minutes of reperfusion following the lethal ischaemic period. This concentration of rapamycin has been shown to inhibit p70S6K phosphorylation in the isolated perfused rat heart;([530])

(6) ***IPC + Chel:*** IPC-treated hearts (n=6) were given chelerythrine (5 µmol/L, a PKC inhibitor) for the first 15 minutes of reperfusion following the lethal ischaemic period. This concentration of chelerythrine has been shown to inhibit PKC phosphorylation in the isolated perfused rat heart;([898])

(7) ***LY***: Hearts (n=6) were given LY 294008 for the first 15 minutes of reperfusion;

(8) ***PD:*** Hearts were given PD 98059 for the first 15 minutes of reperfusion;

(9) ***RAPA:*** Hearts were given rapamycin for the first 15 minutes of reperfusion;

(10) ***Chel:*** Hearts were given chelerythrine for the first 15 minutes of reperfusion;

Figure 6.1: ***Experimental Protocols Investigating the Role of Kinases at the Time of Reperfusion in Preconditioning-Induced Protection.*** LY-LY 294008, PD-PD 98059, RAPA-Rapamycin, Chel- chelerythrine, TTC-tetrazolium.

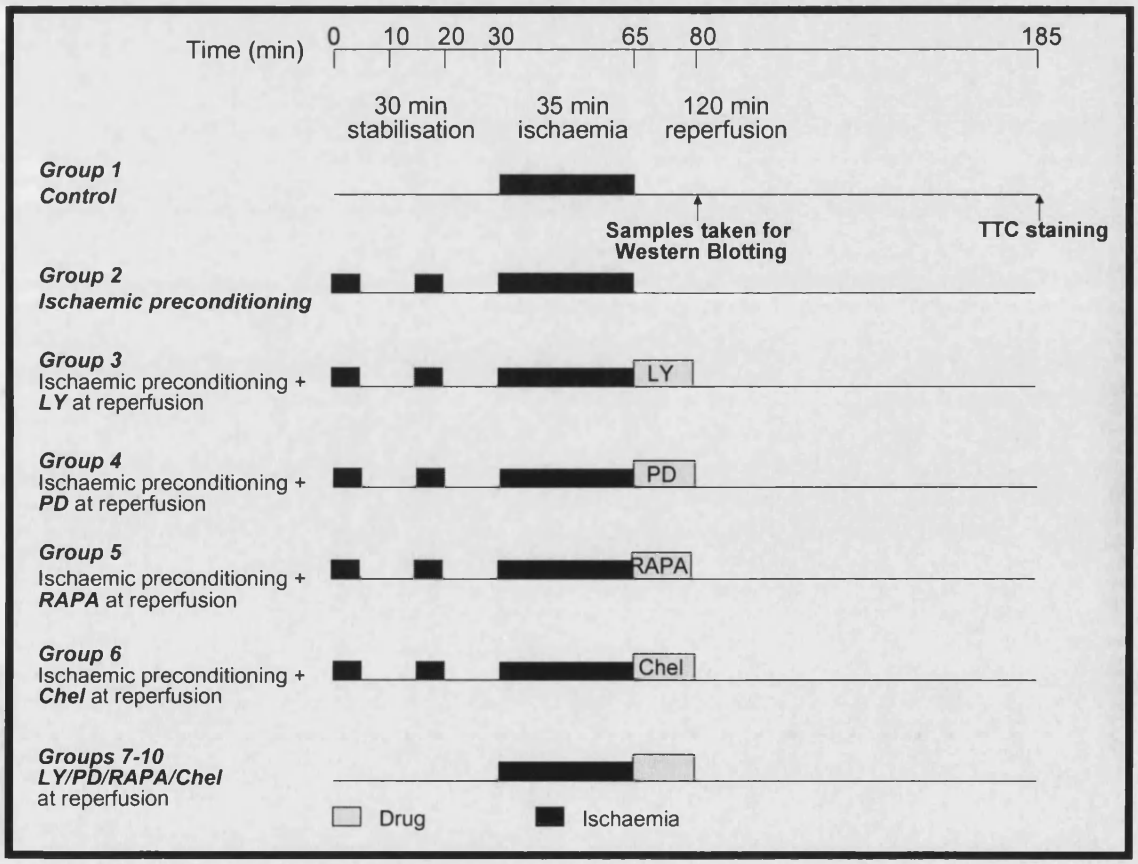
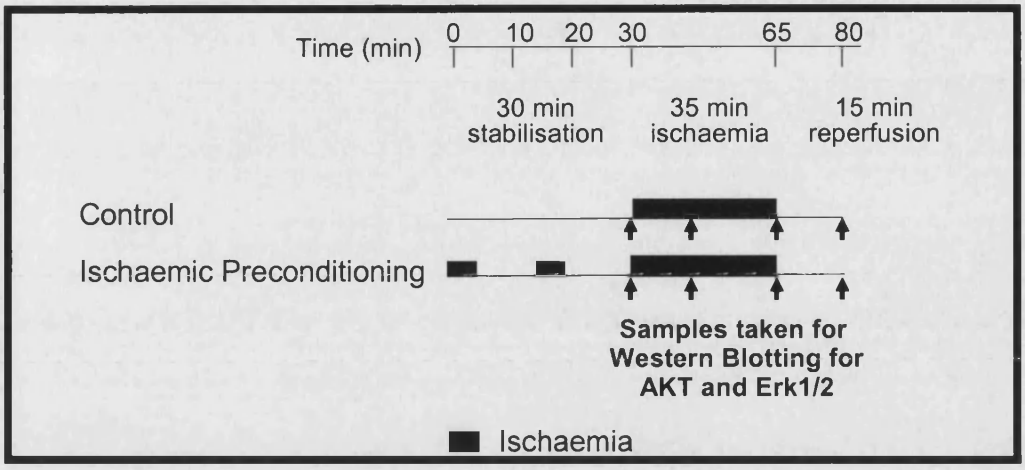


Figure 6.2: ***Experimental Protocols Outlining the Time-Points for Obtaining Tissue Samples for Determination of Akt and Erk1/2 Phosphorylation***



6.3.4 Measuring Akt/Erk1/2/p70S6K phosphorylation using Western blotting

6.3.4.1 Time-Course of Akt and Erk1/2 Phosphorylation Induced by IPC

Control hearts and IPC-treated hearts were subjected to ischaemia-reperfusion and tissue samples were taken from the region-at-risk and collected as outlined in section 3.8.1 at the following four time-points (n=6 at each time-point) for subsequent Western blot analysis for Akt and Erk1/2 phosphorylation (see figure 6.2):

(1) following the period of stabilisation or the IPC-treatment protocol;

(2) after 15 minutes of lethal ischaemia;

(3) at the end of the 35 minute ischaemic period, and

(4) after 15 minutes of reperfusion,

6.3.4.2 Akt, Erk1/2, and p70S6K Phosphorylation at Time of Reperfusion Induced by Ischaemic Preconditioning

Tissue samples were collected after 15 minutes of reperfusion, from hearts undergoing the experimental protocols as outlined in figure 6.1, according to the method described in section 3.8.1. Following protein extraction as outlined in section 3.8.2, protein samples were electrophoresed and probed using antibodies for phospho-Erk1/2 (Thr202/Thr204), phospho-Akt (Ser473), phospho-p70S6 kinase (Thr389) and phospho-p70S6 kinase (Thr421/Ser424), and were used in accordance with the instructions issued by the manufacturer, Cell Signalling (Hitchin, Kent), according to the method employed in section 3.8.5.

6.3.5 Results

6.3.5.1 Exclusions

We used 56 male Sprague-Dawley rat hearts for the infarct size experiments of which 4 were excluded owing to poor function during stabilisation (see section 3.2.2 for exclusion criteria).

6.3.5.2 Animal and Haemodynamic Data

Animal and heart weights and risk zone volumes were similar in the experimental groups (see table 6.1). Baseline data relating to cardiac function (assessed by the rate-pressure-product, RPP) and coronary flow before the lethal ischaemic period, were similar in the experimental groups (see tables 6.2, 6.3). During regional ischaemia, coronary flow and RPP decreased to a similar extent in both groups. An increase in cardiac function and coronary flow upon reperfusion was indicative of successful re-flow.

Table 6.1 Characteristics of Animals in Treatment Groups

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
1. Control	6	380±7	2.32±0.08	0.568±0.062
2. IPC	6	340±10	1.87±0.06	0.395±0.026
3. IPC + LY	6	338±12	1.79±0.16	0.477±0.048
4. IPC + PD	6	355±10	1.95±0.12	0.433±0.023
5. IPC + RAPA	6	333±16	1.85±0.13	0.503±0.032
6. IPC + Chel	6	337±7	1.87±0.03	0.423±0.044
7. LY	4	335±10	1.83±0.12	0.533±0.031
8. PD	4	346±16	1.84±0.13	0.480±0.037
9. RAPA	4	393±3	2.11±0.19	0.550±0.039
10. Chel	4	333±3	1.67±0.04	0.391±0.026

Values are mean±SEM. *P<0.001 compared with control.

Table 6.2 Rate Pressure Product (x 10^3mmHg/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	23.1±3.5	27.4±1.4	11.8±2.2	13.3±2.2	16.0±1.6	10.6±0.6
2. IPC	26.6±2.9	19.3±6.1	10.7±2.6	14.5±2.8	20.9±1.6	14.8±1.5
3. IPC + LY	34.9±5.3*	31.4±3.7	12.3±2.2	12.4±1.8	15.2±1.7	8.6±1.5
4. IPC + PD	28.7±3.4	26.5±2.3	13.5±2.4	15.4±2.3	17.3±2.2	9.3±1.4
5. IPC+RAPA	26.5±3.0	20.6±3.2	9.9±1.7	12.8±2.8	16.0±2.7	11.4±2.1
6. IPC + Chel	23.5±1.1	18.7±4.4	9.4±2.9	16.4±3.0	19.8±4.4	10.6±2.9
7. LY	29.9±3.8	27.9±3.7	11.1±1.8	15.2±3.0	12.1±1.4	13.4±2.5
8. PD	27.2±2.4	25.6±2.3	10.6±1.4	12.4±2.3	14.4±1.7	11.4±2.4
9. RAPA	22.8±3.3	26.4±1.0	10.2±2.4	9.0±1.7	14.7±3.5	8.4±2.9
10 Chel	33.8±0.9*	34.4±3.1*	22.1±5.7	18.7±1.8	23.2±4.0	16.2±0.3

Values are mean±SEM. *P<0.05 compared with control.

Table 6.3 Coronary Flow Rate (ml/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	21.2±1.1	23.5±0.3	12.0±1.2	11.6±0.9	19.8±1.6	14.2±3.0
2. IPC	19.5±1.7	19.0±1.4	7.0±0.6	6.5±0.6	11.5±1.9	6.8±0.8
3. IPC + LY	21.3±1.2	22.8±1.7	11.2±0.9	8.8±1.1	12.3±0.9	10.5±2.2
4. IPC + PD	20.3±1.4	22.4±2.5	8.4±1.3	7.5±1.3	11.4±1.0	8.4±1.4
5. IPC+RAPA	19.8±1.7	21.5±2.6	9.0±1.5	10.2±1.8	12.8±1.3	8.3±0.8
6. IPC + Chel	23.0±0.6	17.3±3.3	8.3±1.7	8.1±2.6	20.3±1.7	9.3±0.7
7. LY	23.5±0.9	22.3±1.3	12.0±1.1	11.7±2.0	16.0±1.1	12.3±2.6
8. PD	22.3±1.9	24.3±2.5	13.4±2.1	11.3±1.8	10.2±1.2	7.4±1.4
9. RAPA	18.0±2.0	18.0±2.0	7.9±0.2	7.0±1.0	8.5±0.5	8.5±2.5
10 Chel	19.3±2.4	20.8±2.0	13.5±3.2	13.8±4.2	21.2±1.1	11.5±1.8

Values are mean±SEM. *P<0.005 compared with control.

6.3.5.3 Western Blot Data

IPC Induces a Biphasic Response in Akt and Erk1/2 Phosphorylation

The IPC stimulus resulted in an immediate increase in Akt phosphorylation (relative density of 242.0±31.8 arbitrary units [a.u] in control to 680.7±160.9 a.u with IPC; P<0.05; figure 6.3a), which then declined during the lethal ischaemic period, followed by a second increase at the time of reperfusion (563.7±74.4 with IPC vs 129.7±33.9 in control; P<0.001; figure 6.3a). IPC resulted in a similar biphasic response in Erk1/2 phosphorylation: during the preconditioning phase (540.3±130.2 in control to 804.2±165.4 with IPC; P<0.05; figure 6.3b) and at the time of reperfusion (834.3±14.1 with IPC vs 275.8±20.8 in control; P<0.001; figure 6.3a).

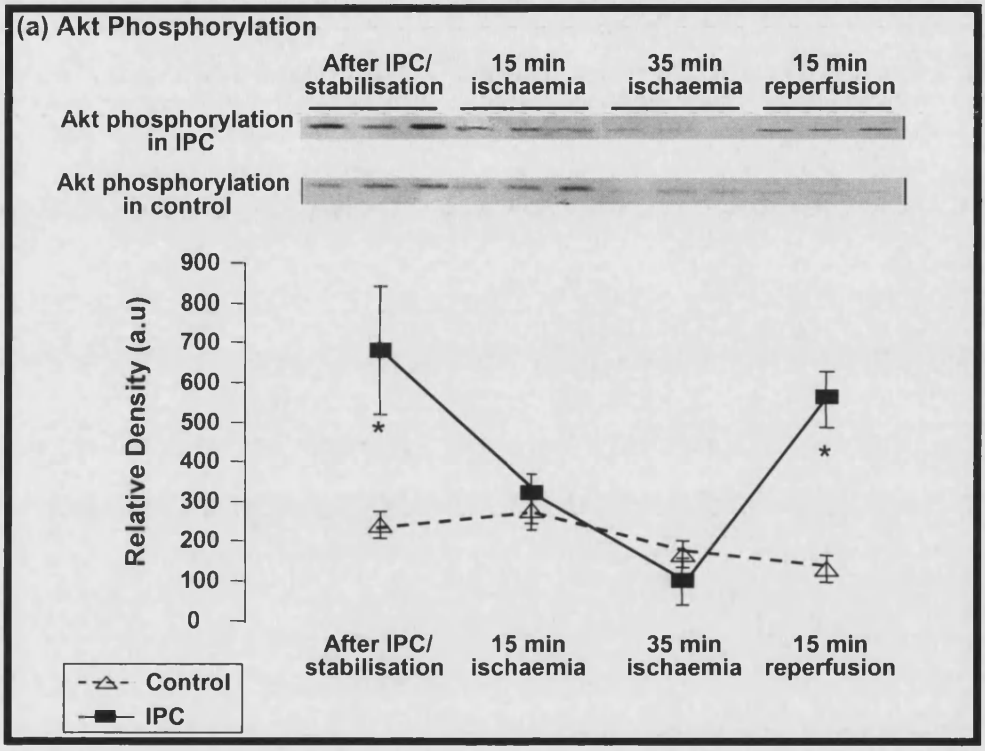
The Presence of LY 294002 or PD 98059 at the Time of Reperfusion Abrogates the IPC-Induced Phosphorylation of Akt, Erk1/2 and p70S6K

In addition to inducing the phosphorylation of Akt and Erk1/2 after 15 minutes reperfusion (figure 6.4), IPC also resulted in the phosphorylation of p70S6K, the kinase downstream of Akt and Erk1/2, at Thr389, the site phosphorylated by Akt-mTOR (2745.0±454.4 with IPC vs 746.7±219.1 in control; P<0.01; figure 6.4) and at Thr421/Ser424, the sites phosphorylated by Erk1/2 (1906.0±110.1 with IPC vs 450.0±34.2 in control; P<0.01; figure 6.5).

The presence of LY (the PI3K inhibitor) during the first 15 minutes of reperfusion abrogated the IPC-induced phosphorylation of Akt (115.7±15.6 with IPC+LY vs 891.3±99.2 with IPC; P<0.01; figure 6.4), and p70S6K at (Thr389) (472.7±89.8 with IPC+LY vs 2745.0±454.4 with IPC; P<0.01; figure 6.4). The presence of PD (the Erk1/2 inhibitor) during the first 15 minutes of reperfusion abrogated the IPC-induced phosphorylation of Erk1/2 (543.0±10.9 in IPC+PD vs 1121.8±78.9 in IPC; P<0.01; figure 6.5) and p70S6K at (Thr421/Ser424) (556.7±189.7 in IPC+PD vs 1906±110.1 in IPC; P<0.01; figure 6.5). The presence of LY 294002 in control hearts at the time of reperfusion, did not influence Akt phosphorylation (relative density 72.0±43.7 a.u with LY vs 280.7±58.4 a.u in control; P=NS; figure 6.4), although it did decrease p70S6K phosphorylation (at Thr389, relative density 149.3± a.u with LY vs 746.7± 219.1 a.u in control; P<0.05; figure 6.4). The presence of PD 098059 at the time of reperfusion in control hearts, did not influence either Erk1/2 phosphorylation (relative density 454.5±143.1 a.u with PD vs 334.3±67.3 a.u in control; P=NS; figure 6.5) or p70S6K phosphorylation (at

Thr421, Ser 424, relative density 872.0±309.1 a.u with PD vs 450.0±34.2 a.u in control; P=NS; figure 6.5).

Figure 6.3: Time-Course of Akt and Erk1/2 Activation in Ischaemic Preconditioning. Representative Western blots and relative densitometry, depicting the time-course of (a) Akt and (b) Erk1/2 phosphorylation, in control and ischaemic preconditioned (IPC) rat hearts. IPC induces two phases of Akt and Erk1/2 phosphorylation, the first occurring immediately following the IPC protocol, and the second occurring at reperfusion. (N=6 at each time-point per treatment group.*P<0.05 compared to control)



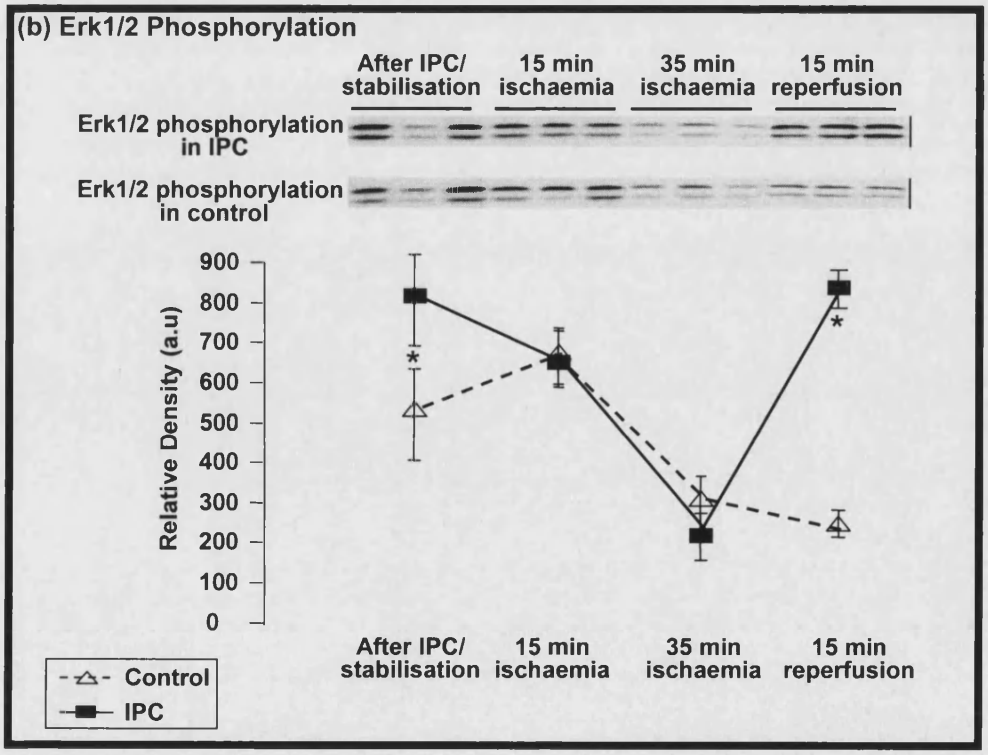


Figure 6.4: Western Blots of Akt and p70S6K Phosphorylation. Representative Western blots and relative densitometry showing that inhibiting PI3K, using LY 294008, abolishes the IPC-induced phosphorylation of Akt and p70S6K at Thr 389 (the site phosphorylated by Akt)(N=6 per group.*P<0.05 compared to control).

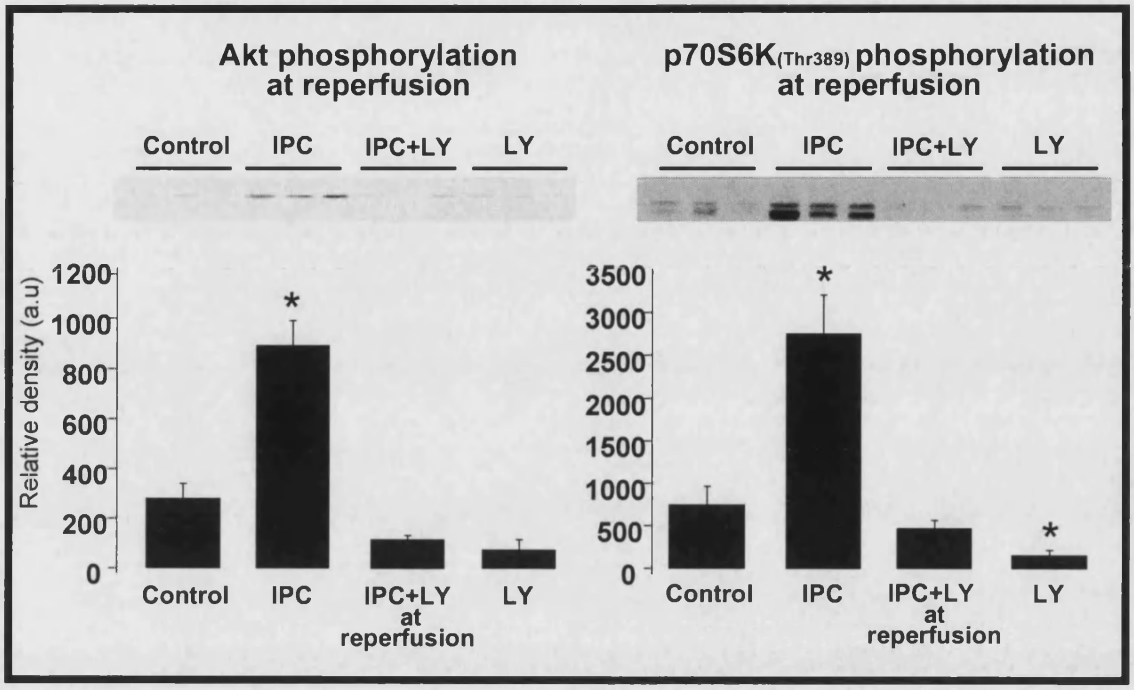
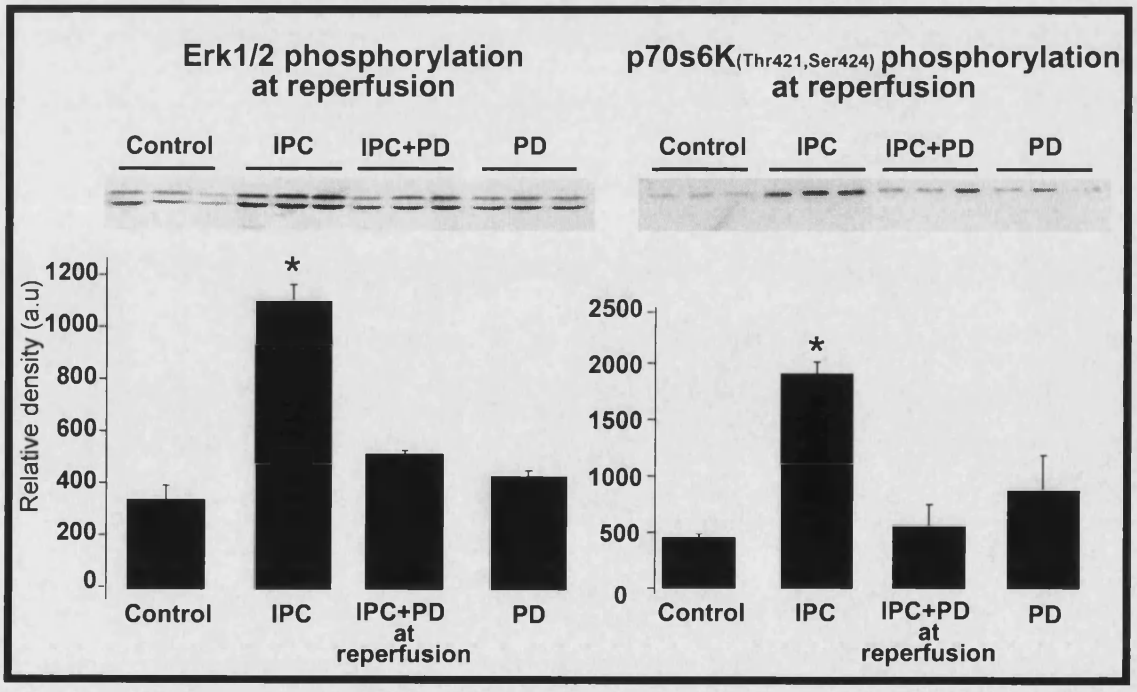


Figure 6.5: Western Blots of Erk1/2 and p70S6K Phosphorylation. Representative Western blots and relative densitometry showing that inhibiting MEK1/2, using PD 98059, abolishes the IPC-induced phosphorylation of Erk1/2 and p70S6K at Thr421/Ser424 (the sites phosphorylated by Erk1/2)(N=6 per group.*P<0.05 compared to control).



'Cross-talk' between the PI3K-Akt and MEK1/2-Erk1/2 Kinase Cascades at the Time of Reperfusion

This was a surprising and interesting finding of the study, which did not form one of our initial objectives of the study. Inhibiting MEK1/2 in control hearts, during the reperfusion phase, using PD 98059 alone, resulted in the phosphorylation of Akt (785±78 a.u with PD alone vs 281±58 a.u in control; P<0.01; figure 6.6) and a non-significant increase in phosphorylation of the downstream p70S6K at Thr389 (356±52 a.u with PD alone vs 163±41 a.u in control; P=NS; figure 6.6). Inhibiting MEK1/2 at reperfusion in IPC-treated hearts did not increase Akt phosphorylation above that observed in hearts that had only received IPC treatment (914±18 a.u in IPC+PD vs ± 891±99 a.u in IPC; P=NS; figure 6.6).

Conversely, inhibiting PI3K in control hearts, during the reperfusion phase, using LY 294002, resulted in the phosphorylation of Erk1/2 (913±225 a.u with LY alone vs 334±67 a.u in control; P<0.01; figure 6.7) and the downstream p70S6K at Thr421, Ser424 (1260±60 a.u with LY alone vs 450±34 a.u in control; P<0.01; figure 6.7). Interestingly, in this case, the inhibition of PI3K at the time of reperfusion resulted in the activation of Erk1/2 over and above that induced by IPC alone (1442±134 a.u in IPC+LY vs ± 1122±79 a.u in IPC; P<0.001; figure 6.7).

Figure 6.6: 'Cross-Talk' Between PI3K-Akt and MEK1/2-Erk1/2. Representative Western blots and relative densitometry showing that inhibiting MEK1/2, using PD 98059, results in phosphorylation of Akt but not p70S6K at Thr389 (the site phosphorylated by Akt) The lower band of the blot represents p70S6K. (N=6/group.*P<0.05 compared to control).

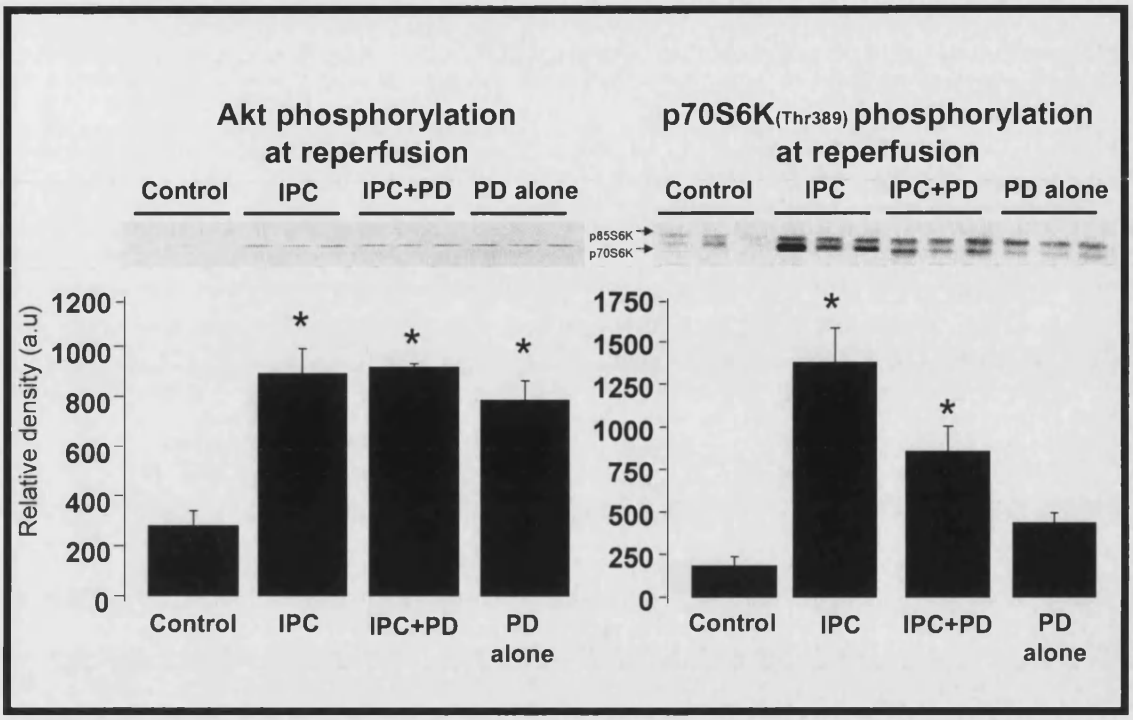
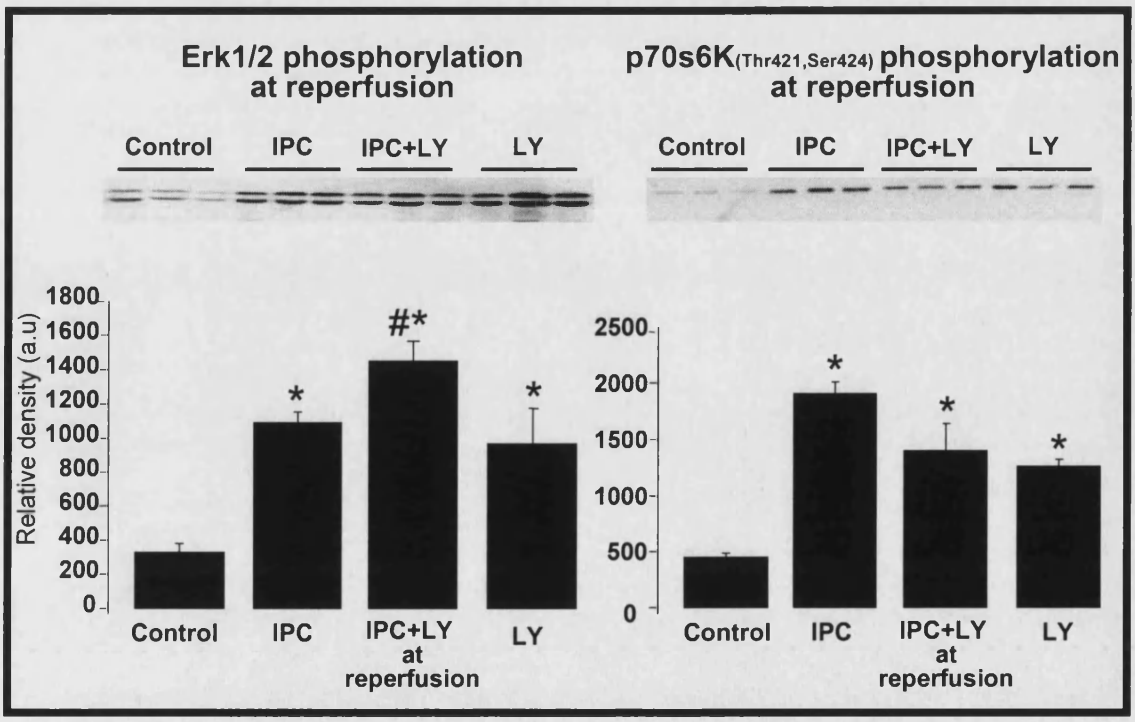


Figure 6.7: Cross-Talk Between PI3K-Akt and MEK1/2-Erk1/2. Representative Western blots and relative densitometry showing that inhibiting Akt, using LY 294008, results in phosphorylation of Erk1/2 and p70S6K at Thr421/Ser424 (the sites phosphorylated by Erk1/2)(N=6/group.*P<0.05 compared to control).



6.3.5.4 Infarct Data

Inhibiting Either Akt or Erk1/2 Phosphorylation at the Time of Reperfusion Abrogates the IPC-Mediated Reduction in Infarct Size

IPC reduced infarct size (56.9±5.7% in control vs 20.9±3.6% with IPC; P<0.01; figure 6.8). The presence of LY (the PI3K inhibitor) or PD (the MEK1/2 inhibitor) for the first 15 minutes of reperfusion, abrogated the IPC-induced reduction in infarct size (46.3±5.8% with IPC+LY, 49.2±4.0% with IPC+PD vs 20.9±3.6% with IPC; P<0.01; figure 6.8), suggesting that phosphorylation of Akt and Erk1/2, in the first few minutes of reperfusion, are required for IPC-induced protection.

The 'Cross-talk' Demonstrated Between the PI3K-Akt and Raf1-MEK1/2-Erk1/2 Kinase Cascades is not Associated with Protection

In order to determine whether the PD 98059-induced phosphorylation of Akt and the LY 294008-induced phosphorylation of Erk1/2 were associated with protection at the time of reperfusion, we examined the effect of these kinases on infarct size. Inhibiting PI3K, using LY 294002 for the first 15 minutes of reperfusion in control hearts did not influence infarct size (43.1±7.2% with LY vs 46.3±5.8% in control; P=NS; figure 6.8), suggesting that the LY 294002-induced Erk1/2 phosphorylation is not sufficient in itself to induce protection. Similarly, inhibiting MEK1/2, using PD 98059 for the first 15 minutes of reperfusion in control hearts did not influence infarct size (57.7±7.0% in PD vs 46.3±5.8% in control; P=NS; figure 6.8), suggesting that the PD 98059-induced Akt phosphorylation is also not sufficient in itself to induce protection.

Inhibiting p70S6K phosphorylation at the time of reperfusion abrogates the IPC-mediated reduction in infarct size

The presence of rapamycin (the p70S6K inhibitor) for the first 15 minutes of reperfusion, abrogated the IPC-induced reduction in infarct size (46.0±7.7% with IPC+RAPA vs 17.8±2.3% with IPC; P<0.01; figure 6.9), implicating the activation of p70S6K as a mediator of IPC-induced protection. Rapamycin given alone at time of reperfusion to control hearts did not influence infarct size (36.0±5.4% with RAPA vs 46.3±5.8% in control; P=NS; figure 6.9).

Inhibiting PKC Phosphorylation at the Time of Reperfusion Abrogates the IPC-Mediated Reduction in Infarct Size

The presence of chelerythrine (the PKC inhibitor) for the first 15 minutes of reperfusion, also abrogated the IPC-induced reduction in infarct size (42.6±5.3% with IPC+Chel vs 17.8±2.3% with IPC; P<0.01; figure 6.9). Chelerythrine given alone at time of reperfusion to control hearts did not influence infarct size (38.0±1.1% with Chel vs 46.3±5.8% in control; P=NS; figure 6.9).

Figure 6.8: Inhibiting Either Akt or Erk1/2 Phosphorylation at Reperfusion Abrogates IPC-Induced Protection. Inhibiting PI3K (using LY294002, LY) or MEK1/2 (using PD98059, PD) for the first 15 minutes of post-ischaemic reperfusion, abrogates the ischaemic preconditioned (IPC)-induced reduction in infarct-risk volume ratio.(N=6/group.*P<0.01).

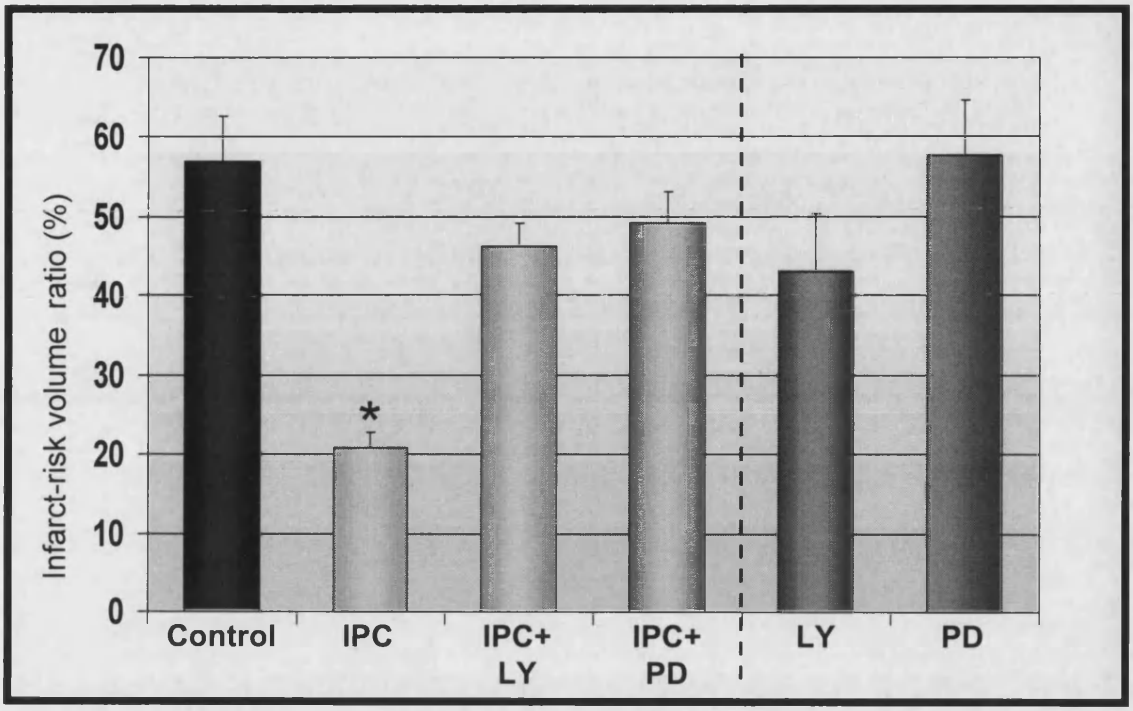
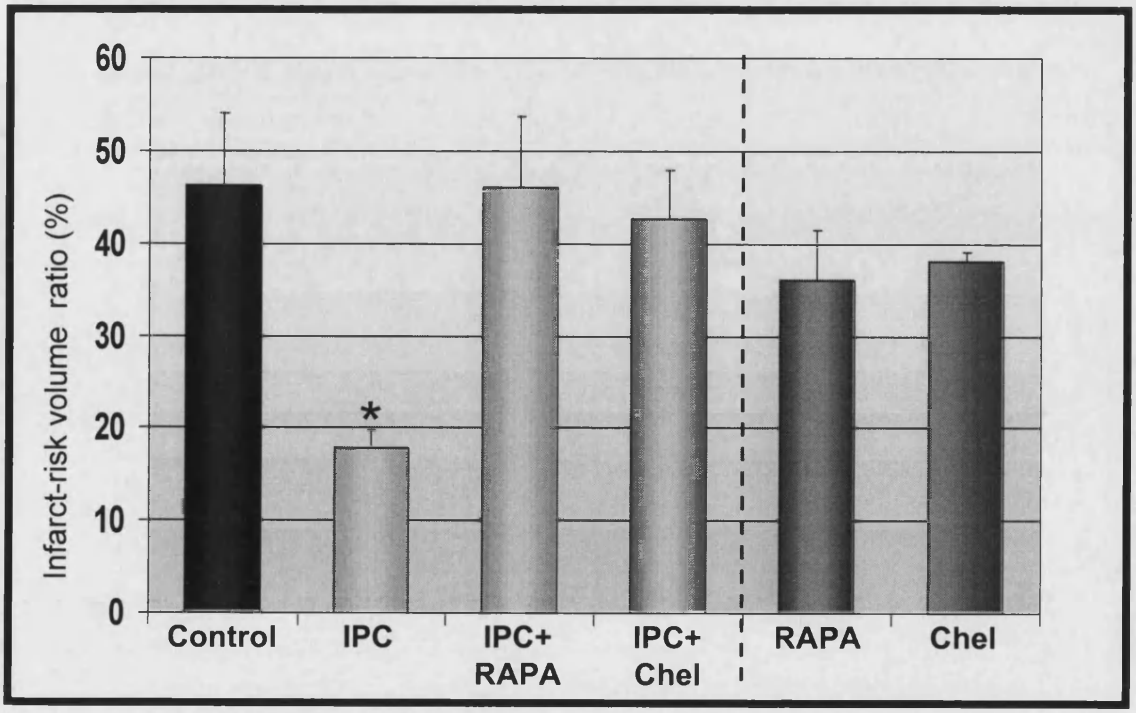


Figure 6.9: Inhibiting Either p70S6K or PKC Phosphorylation at Reperfusion Abrogates IPC-Induced Protection. Inhibiting p70S6K (using rapamycin, RAPA) or PKC (using chelerythrine, Chel) for the first 15 minutes of post-ischaemic reperfusion, abrogates the ischaemic preconditioned (IPC)-induced reduction in infarct-risk volume ratio.(N=6/group.*P<0.01).



6.3.6 Discussion

The main findings of this section of the study were as follows: (1) We demonstrated for the first time that IPC induces the activation of both the PI3K-Akt-p70S6K and the MEK1/2-Erk1/2-p70S6K signalling cascades at the time of reperfusion, following a lethal ischaemic period. (2) Furthermore, we show that the activation of these kinase cascades at reperfusion are essential to mediate IPC-induced protection, as inhibiting their activation at this time, abrogates their IPC-mediated infarct-limiting effect.

Several studies have demonstrated that the activation of the PI3K-Akt pathway during the preconditioning phase mediates IPC-induced protection,([81;82]) as well as insulin-mediated preconditioning.([83]) Whether MEK1/2-Erk1/2 activation during the preconditioning phase, contributes to IPC-induced protection is less conclusive.([82;193;194;196]) In this scenario the kinase activation takes place during the preconditioning phase and the kinases act as triggers of preconditioning mediating the preconditioning signal to targets situated further downstream the signalling pathway such as ROS and PKC. We confirm in our study that IPC produces phosphorylation of both Akt and Erk1/2 during the preconditioning phase, prior to the index ischaemic period. The absence of kinase activation noted during ischaemia may be due to the lack of ATP.

We demonstrated for the first time, that in addition to inducing the phosphorylation of the PI3K-Akt cascade during the preconditioning phase, IPC appears to induce a second phase of phosphorylation of the PI3K-Akt cascade, at the time of reperfusion, following the lethal ischaemic period. However, this second phase of activation noted at reperfusion could be a continuation of the signal induced prior to ischaemia, and the kinases are reactivated at reperfusion, because ATP is again present.

Furthermore, we demonstrate that IPC induces two phases of Erk1/2 phosphorylation, the first one occurring during the preconditioning phase, followed by a second phase taking place at reperfusion, confirming the findings of a study by Fryer and colleagues,([194]) in which they observed that preconditioning with either ischaemia or a δ-opioid receptor agonist resulted in two phases of Erk1/2 phosphorylation. However, in that study, these authors did not determine whether the IPC-induced Erk1/2 phosphorylation that occurred at reperfusion, was required to mediate IPC-induced protection.

Previous studies have demonstrated that, activating the PI3K-Akt and MEK1/2-Erk1/2 cascades, which together comprise the Reperfusion Injury Salvage Kinase (RISK)-pathway, at the time of reperfusion, protects the heart, with numerous growth factors initiating protection when given at reperfusion, through the up-regulation of this pathway.([519]) The intriguing implication of the present study, is that IPC may also recruit the PI3K-Akt and MEK1/2-Erk1/2 cascades, during the reperfusion phase to mediate its protection.

Further studies are required to ascertain, why an IPC stimulus should result in the activation of these pro-survival kinases cascades at the time of reperfusion. We propose that PKC and reactive oxygen species (ROS) may be important candidates in this scheme, as they have both been implicated as early mediators of the preconditioning signal,([72;173]) and both have been demonstrated to activate these kinase cascades.([223;327;899])

In support of PKC as a mediator of kinase activation at the time of reperfusion, we found that inhibiting PKC activation at the time of reperfusion also abolished IPC-induced protection suggesting the role of PKC as a mediator of IPC-induced protection at the time of reperfusion. However, further studies are required to determine whether the phosphorylation of Akt and Erk1/2 are dependent on PKC, before one can implicate the latter as a mediator of kinase activation at the time of reperfusion in the setting of IPC.

In support of ROS as a mediator of kinase activation at the time of reperfusion, it is interesting to note that, the phosphorylation of Akt and Erk1/2, that was observed to occur during the preconditioning phase, declined to control levels during the ischaemic phase, and increased again during the reperfusion phase. This finding suggests that the act of 'reperfusion' itself may be important in mediating the phosphorylation of Akt and Erk1/2. Reactive oxygen species (ROS) may contribute to the activation of these kinase cascades that occur following the IPC treatment and at time of reperfusion, based on the fact that: (1) ROS have been shown to be produced during the preconditioning and reperfusion phases, but not during the lethal ischaemic period;([900]) and (2) ROS have been shown to activate the kinase cascades at the time of reperfusion.([223])

We were also intrigued by the temporal relationship between the two observed phases of Akt and Erk1/2 kinase phosphorylation induced by an IPC stimulus. We propose that the first phase of IPC-induced kinase phosphorylation observed prior to ischaemia, may either 'prime' the kinase cascade or change the intracellular distribution of these kinases, such that at the time of reperfusion, the second phase of kinase phosphorylation is amplified and mediates the

protection against lethal reperfusion-induced injury. In this regard, Fujio and colleagues observed in mouse hearts that the increased Akt activity induced by IGF-1 over-expression, was amplified at the time of reperfusion.([542])

Therefore, both the phases of kinase phosphorylation appear to be required to mediate IPC-induced protection, as inhibiting the kinase cascades either during the preconditioning phase,([81;194]) or at the time of reperfusion, as demonstrated in the current study, abolishes IPC-induced protection.

'Cross-talk' Between the PI3K-Akt and MEK1/2-Erk1/2 Kinase Cascades and the Role of p70S6K

A surprising finding from this part of the study, was the observation that the PI3K-Akt and Raf-MEK1/2-Erk1/2 kinase cascades, which are activated at the time of reperfusion in response to ischaemic preconditioning, exhibit 'cross-talk'.

Cross-talk between these kinase cascades had not been investigated in the setting of myocardial ischemia-reperfusion. We found that, inhibiting PI3K, using LY 294002, at the time of reperfusion resulted in the activation of the MEK1/2-Erk1/2-p70S6K kinase cascade, and that the inhibition of MEK1/2 with PD 98059 at the time of reperfusion resulted in the activation of the PI3K-Akt kinase cascade, but not p70S6K. Interestingly, this 'cross-talk' between the kinase cascades was not associated with protection, in terms of a reduction in infarct size, which suggests that the PI3K-Akt phosphorylation induced by PD 98059 (the MEK1/2 inhibitor) and the MEK1/2-Erk1/2-p70S6K phosphorylation induced by LY 294008 (the PI3K inhibitor) were insufficient in themselves to induce protection, and indicates that both the kinase cascades may be required to be activated at the time of reperfusion to mediate IPC-induced protection.

The previous data had demonstrated that inhibiting either the Akt or the Erk1/2 activation that occurred at the time of reperfusion, using LY 294008 or PD 98059, respectively, completely abrogated IPC-induced protection, also indicating the requirement for both kinase cascades to mediate protection. The fact that both cascades appear to work in concert to mediate IPC-induced protection at reperfusion, suggests they may converge on a common distal signalling moiety. A potential candidate in this scheme may be the downstream p70S6K, which is phosphorylated at different sites by the PI3K-Akt and MEK1/2-Erk1/2 cascades.([591;901]) One can postulate that IPC-induced protection may require phosphorylation at both sites of the

p70S6K in order to execute protection.([902]) In this scenario therefore, inhibiting one of the kinase cascades may be able to completely abolish IPC-induced protection.

Activation of the p70S6K may mediate IPC-induced protection via an anti-apoptotic mechanism, in which it phosphorylates and inactivates the pro-apoptotic protein BAD.([581]) In this regard, Jonassen and colleagues have demonstrated that the protection induced by insulin at the time of reperfusion is mediated by activation of the Akt-p70S6K-BAD pathway.([530]) Studies in haematopoietic cells, have also observed the requirement for both kinases cascades to be activated to induce cellular protection,([903]) and interestingly, a study by Muscarella & Bloom suggested that the kinase cascades appeared to converge on p90S6 kinase (RSK).([904]) In the present study, we demonstrated that IPC results in the activation of p70S6K at phosphorylation sites specific for Akt (at Thr 389) and Erk1/2 (at Thr421, Ser424), at the time of reperfusion.([591;901])

We found that the presence of the known p70S6K inhibitor, rapamycin, at the time of reperfusion completely abrogated IPC-induced protection, suggesting that p70S6K may act as the point of convergence for these kinase cascades. However, without including data actually demonstrating rapamycin-mediated inhibition of the IPC-induced phosphorylation of p70S6K, we cannot categorically state that p70S6K is implicated in IPC-induced protection. We speculate that the activation of p70S6K requires the phosphorylation at both Thr 389 (the site phosphorylated by Akt-mTOR) and Thr421, Ser424 (the sites phosphorylated by Erk1/2), which would necessitate the activation of both the PI3K-Akt and MEK1/2-Erk1/2 pathways to mediate IPC-induced protection. Previous studies have demonstrated that signalling through these kinase cascades can converge on another distal target, the pro-apoptotic protein, BAD, the phosphorylation and inactivation of which would mediate cellular survival by an anti-apoptotic mechanism.([574;607;608])

The phenomenon of 'cross-talk' observed between the kinase cascades, in which the inhibition of one kinase cascade results in the activation of the other and vice versa, has been observed in other organ tissue, including the lens([609]) and neuronal cells.([603]) In previous studies, using immortal cell lines, it has been demonstrated that Akt inhibits the Raf-MEK1/2-Erk1/2 kinase cascade by phosphorylation and inactivation of Raf at Ser^{259}.([600;601]) and this inhibitory pathway may be recruited at different stages of cell development,([600]) or vary according to the concentration and type of ligand exposure.([604]) Therefore, the inhibition of the PI3K-Akt pathway may activate the Raf-MEK1/2-Erk1/2 cascade, providing a form of

'compensatory regulation'. This would explain, why the inhibition of PI3K at the time of reperfusion induces the activation of MEK1/2-Erk1/2, as the inhibitory pathway from Akt to Raf-MEK1/2-Erk1/2 is removed.

Whether the corollary occurs, in which the activation of Akt occurring in response to the inhibition of the Raf-MEK1/2-Erk1/2 cascade is the result of the removal of an inhibitory pathway from Erk1/2 on the PI3K-Akt pathway, is unclear at present. We did not find any evidence to suggest that the PI3k-Akt cascade facilitates the Raf-MEK1/2-Erk1/2 kinase cascade as has been shown in previous studies.([602-604]) Nor did we find evidence that MEK1 activation activates the PI3K-Akt-p70S6K pathway as been suggested by several studies.([605;606]) The disparity in findings may rest with the different experimental models used and vary with the experimental conditions.([602])

Interestingly, the 'cross-talk' observed between the two kinase cascades did not appear to be equal in that inhibiting MEK1/2 at the time of reperfusion in control hearts resulted in the phosphorylation of Akt but not p70S6K (at Thr389). Furthermore, inhibiting MEK1/2 at the time of reperfusion in IPC-treated hearts did not result in the phosphorylation of either Akt or p70S6K (at Thr389) to a greater level than that observed in hearts undergoing IPC-alone. In direct contrast, we found that inhibiting PI3K at the time of reperfusion in control hearts resulted in the phosphorylation of Erk1/2 and its downstream p70S6K (at Thr421/Ser424). Furthermore, inhibiting PI3K at the time of reperfusion in IPC-treated hearts resulted in the phosphorylation of Erk1/2 but not p70S6K (at Thr421/Ser424) to a greater level than that observed in hearts undergoing IPC-alone. These findings would suggest that the cross-talk between the two kinase cascades is not balanced and that in the scenario of cellular survival, the PI3K-Akt cascade is the more dominant cascade, with the MEK1/2-Erk1/2 cascade playing a more significant role in mediating growth and hypertrophy.

In conclusion, we report that the activation of the PI3K-Akt and MEK1/2-Erk1/2 kinase cascades that occurs at the time of reperfusion, in response to ischaemic preconditioning, occurs in parallel and that both kinase cascades are required to act in concert to mediate IPC-induced protection. The downstream kinase, p70S6K may be a point of convergence for the PI3K-Akt and MEK1/2-Erk1/2 kinase cascades in mediating IPC-induced protection, although further studies are required to confirm this. These two pathways appear to interact in such a way, that inhibiting one kinase cascade up-regulates the activity of the other pathway, thereby acting as a compensatory safe-guard, ensuring the signal for cellular protection is executed.

Further work is needed to explore the intricate interactions between these two kinase cascades at the time of reperfusion, in the setting of IPC-induced protection.

In this section, we have demonstrated that IPC protects the heart by activating the PI3K-Akt and MEK1/2-Erk1/2 kinase cascades at the time of reperfusion. Interestingly, we also found that these kinase cascades exhibit 'cross-talk' at the time of reperfusion such that inhibiting one kinase cascade up-regulates the other. In addition, it appears that both kinases are activated in response to an IPC stimulus and both are required to mediate protection. However, how these kinases mediate protection at the time of reperfusion is unclear and is the subject of the next section of the study.

6.4 Aim (2):

Determine whether the pro-survival kinases Akt and Erk1/2 protect by inhibiting the prolonged (high-conductance) mPTP opening at the time of reperfusion.

The previous section demonstrated that IPC protects the heart by activating the PI3K-Akt and MEK1/2-Erk1/2 kinase cascades at the time of reperfusion. Previous studies by Yellon's group have demonstrated that activating these kinase cascades at the time of reperfusion protects the heart against lethal reperfusion injury (reviewed in reference ([519])). This part of the study presents provisional data investigating whether these kinases protect the heart by inhibiting mPTP opening at the time of reperfusion.

In this section of the study, we studied the effect of insulin on mPTP opening in the adult rat myocyte using the oxidative stress model of mPTP opening used in section 5.7. Insulin has been demonstrated to protect the heart against lethal reperfusion injury by activating the PI3K-Akt cascade.([530;895]) Importantly, to mediate protection insulin has to be present in the first few minutes of reperfusion,([530]) the time period, when mPTP opening has been demonstrated to occur. Therefore, the insulin-mediated activation of the kinase cascades was used as a surrogate model for the IPC-mediated activation of the kinase cascades.

6.4.1 Materials

Insulin was dissolved in distilled water (Actrapid, Novo Nordisk Pharmaceuticals, Crawley, West Sussex). Tetramethyl rhodamine methyl ester (TMRM, Molecular Probes Inc., Leiden, The Netherlands) were dissolved in dimethyl sulphoxide (DMSO). All other reagents were of standard analytical grade.

6.4.2 Myocyte Model of mPTP Opening

Adult rat myocytes were isolated from male Sprague-Dawley rats according to the method in described in section 3.5. Isolated myocytes were seeded onto 25-mm round cover-slips according to the method in described in section 3.6. Opening of the mPTP in adult rat myocytes was induced and detected using a cellular model of oxidative stress, as described in section 5.7.

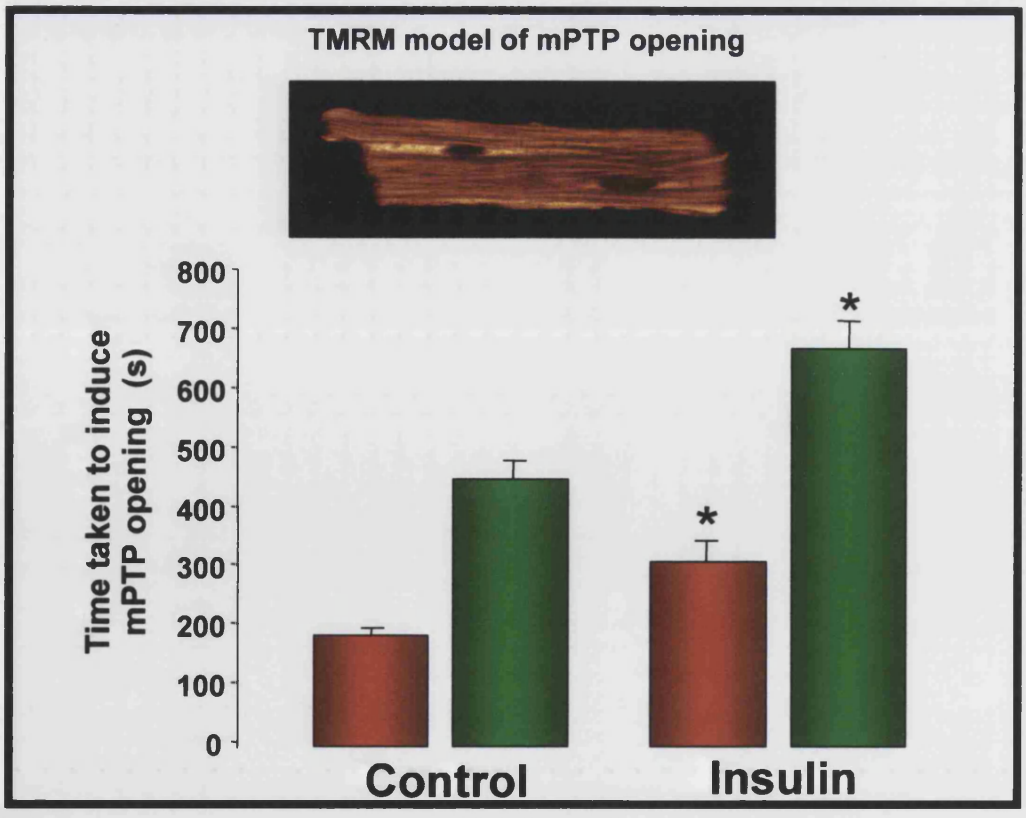
6.4.3 Experimental Protocols for Myocyte Studies

After loading with TMRM in the restoration buffer, cells were randomly assigned to one of the following treatment groups. Cells were incubated for 20 minutes with insulin at 37°C and then subjected to the TMRM-oxidative stress protocol: (1) ***Control***-incubation with restoration buffer alone (n=18) and (2) ***Insulin*** (0.3 mU/ml, n=12). This concentration of insulin has been shown to be cardio-protective in the rat heart;([530])

6.4.4 Results

Insulin treatment increased the illumination time required to induce global mitochondrial membrane depolarization from 181.9±14.7 seconds in control to 294.5±15.8 seconds in insulin-treated cells, (P<0.001, figure 6.10). The times taken to induce rigour was also extended from 473.3.±21.2 seconds in control to 688.2±28.4 seconds (P<0.001, figure 6.10). These results suggest that insulin treatment acts to inhibit the opening of the mPTP induced by oxidative stress, resulting in protection from oxidative stress-induced rigour contracture.

Figure 6.10: The Effect of Insulin on mPTP Opening. Insulin treatment prolongs the duration of laser-induced oxidative stress required to cause both mPTP opening and rigour contracture in TMRM-loaded myocytes. (Values are Mean±SEM. *P<0.001 compared to control)



6.4.5 Discussion

In this section, we have demonstrated that insulin treatment protects myocytes against oxidative stress by inhibiting mPTP opening. Insulin has previously been demonstrated to protect the heart by activating the PI3K-Akt kinase cascade.([530]) This forms provisional data suggesting that activation of the kinase cascades may protect the heart by inhibiting mPTP opening at the time of reperfusion. These results are preliminary and further studies are required to directly demonstrate that IPC inhibits mPTP opening at the time of reperfusion by activating these kinase cascades.

Interestingly, there are two possible mechanisms through which the PI3K-Akt pathway could potentially mediate inhibition of mPTP opening: (1) Activation of the PI3K-Akt kinase cascade has been demonstrated to activate eNOS.([585]) Gao and colleagues have demonstrated that insulin protects at reperfusion by activating the PI3K-Akt-eNOS cascade.([531]) Nitric oxide has been demonstrated to inhibit mPTP opening,([489]) and data from Lemaster's group suggests that the nitric oxide donor, SNAP, given at the time of reoxygenation can protect myocytes against anoxia-reoxygenation injury by inhibiting mPTP opening (unpublished data, 2003). (2) The pro-apoptotic factors, Bax and BAD have been demonstrated to execute apoptosis by opening the mPTP.([751]) Activation of Akt results in the phosphorylation of Bax which prevents its translocation to the mitochondria([575;576]) and the phosphorylation of BAD, which prevents it from translocating to the mitochondria.([574]) Therefore, activation of Akt could potentially inhibit mPTP opening by neutralising the effect of pro-apoptotic factors.

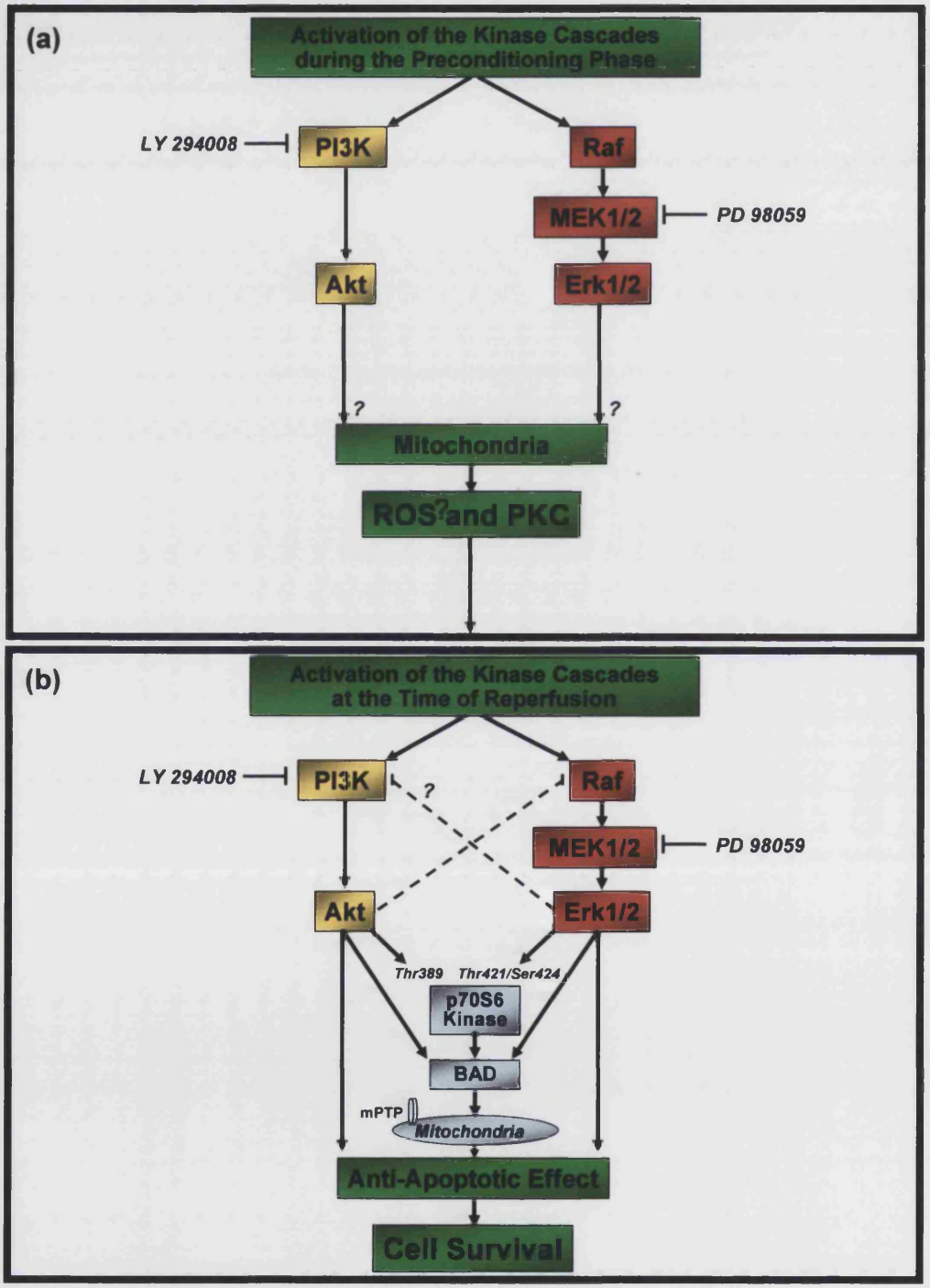
6.5 Discussion and Conclusion

In this section, we have shown for the first time that IPC results in the activation of the P13K-Akt-p70S6K and MEK1/2-Erk1/2-p70S6K kinase cascades *during the early reperfusion phase,* following a lethal ischaemic insult, and we demonstrated that the activation of these kinase cascades at this time are essential to mediate IPC-induced protection. Importantly, we have demonstrated the importance of the reperfusion phase for IPC-induced protection and show that by activating the pro-survival PI3K-Akt and MEK1/2-Erk1/2 kinase cascades at the time of reperfusion, IPC is able to modify events that occur at reperfusion, and protect the heart against lethal reperfusion injury. Preliminary data suggests that PKC activation at the time of reperfusion is also required for IPC-induced protection. Further studies are required to determine whether PKC is the potential up-stream activator of these kinase cascades at the time of reperfusion in the setting of IPC.

Interestingly, we find that the activation of the PI3K-Akt and MEK1/2-Erk1/2 kinase cascades that occurs at the time of reperfusion, in response to ischaemic preconditioning, occurs in parallel and that both are required to act in concert by convergence on p70S6K to induce IPC-induced protection. Furthermore, these two pathways appear to interact in such a way, that inhibiting one kinase cascade up-regulates the activity of the other pathway, thereby acting as a compensatory safe-guard, ensuring the signal for cellular protection is executed.

We have preliminary data to suggest that activating the kinase cascades using insulin may protect the heart by inhibiting mPTP opening. However, further studies are required to demonstrate directly that IPC inhibits mPTP opening at the time of reperfusion via the activation of PI3K-Akt and MEK1/2-Erk1/2. Figure 6.11 depicts the biphasic activation of these kinase cascades in the setting of IPC and demonstrates the possible interaction between the two kinase cascades in mediating their cardio-protective effect.

Figure 6.11 (a-b): Biphasic Activation of the PI3K-Akt MEK1/2-Erk1/2 Kinase Cascades in Ischaemic Preconditioning. (a) The kinase cascades are first activated during the preconditioning phase where they act as preconditioning 'triggers' relaying the preconditioning signal to downstream signalling components such as ROS and PKC. (b) At reperfusion, the kinase cascades are re-activated possibly by ROS and PKC. The kinase cascades then recruit several anti-apoptotic mechanisms such as the inactivation of the pro-apoptotic protein, BAD and the inhibition of mPTP opening which mediate cellular protection. The existence of inhibitory pathways between the kinase cascades, may explain why the inhibition of one cascade using either LY294008 or PD98059 results in the activation of the other and vice versa. Convergence on the downstream kinase p70S6K and the pro-apoptotic factor, BAD, may explain the requirement for both kinase cascades to be activated to mediate IPC-induced protection.



This part of the study has focused on the role of the pro-survival PI3K-Akt and Erk1/2 kinase cascades in mediating protection at the time of reperfusion in the setting of IPC. However, it is interesting to note that the same kinase cascades are activated during the preconditioning phase,([81;82;194]) as we demonstrated in the time course of kinase activation in IPC. Therefore, the kinase cascades may constitute a common pathway of cardio-protection, mediating the protection associated with both IPC and agents which have been demonstrated to protect against lethal reperfusion injury by activating these kinase cascades at the time of reperfusion.([519]) Evidence in support of this proposition, is provided by the fact that agents which precondition, such as bradykinin and AMP579,([596;597]) also induce protection when given at reperfusion.([494;524]). Conversely, agents which have been demonstrated to protect at reperfusion by activating the RISK-pathway, such as insulin, urocortin and CT-1 have also been shown to precondition the myocardium.([83;391;598]) The evidence would tend to suggest that the pro-survival kinase cascades may therefore constitute a common pathway, mediating the cardio-protection induced by IPC on the one hand, as well as protecting the myocardium through their recruitment at the time of reperfusion, on the other hand.

Pharmacological manipulation and up-regulation of the pro-survival kinase cascades, during the early reperfusion phase, may therefore provide a novel approach to salvaging viable myocardium, and provide new treatment strategies that can be used as an adjunct to thrombolytic therapy in the treatment of acute myocardial infarction.

The next part of the thesis examines the mPTP as a potential mediator of preconditioning-induced protection.

Chapter Seven

THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE AS A MEDIATOR IN MYOCARDIAL PRECONDITIONING

7.1 INTRODUCTION

Until this point of the thesis, we have been examining the role of the mPTP as a mediator of cell death in the setting of ischaemia-reperfusion injury and its modification by myocardial preconditioning. In this scenario, the mPTP assumes a prolonged high-conductance, non-selective open conformation, which results in apoptotic and necrotic cell death as outlined in section 1.5.4. The previous chapters have demonstrated how inhibiting this form of mPTP opening at the time of reperfusion using either pharmacological mPTP inhibitors or by myocardial preconditioning is a powerful mechanism of protection.

In addition to this form of mPTP opening, which leads to cell death, numerous studies describe the existence of a low-conductance (<300 Da) ion-selective (Ca^{2+}, H^{+} and K^{+}) state of mPTP opening (also called transient mPTP opening or 'mPTP flicker'), which may occur under physiological circumstances, and does not lead to cell death (see section 1.5.6).([9;10;647;648;692;817;825])

Interestingly, transient (low-conductance) mPTP opening has some features in common with the mechanisms implicated in triggering/mediating preconditioning:

(1) Preconditioning has been demonstrated to attenuate mitochondrial Ca^{2+} loading.([52;239;257;258;261;262]) This form of mPTP opening may mediate mitochondrial Ca^{2+} efflux thereby emptying mitochondria of Ca^{2+};([674;834;835])

(2) ROS released by the mitochondria into the cytosol in response to an preconditioning stimulus are believed to mediate preconditioning by activating kinases such as PKC.([49;67;72]) Certain types of ROS such as superoxide are membrane impermeable([905]) and may require a channel to exit mitochondria into the cytosol. This form of mPTP opening has been demonstrated to mediate mitochondrial ROS release into the cytosol;([840]) Conversely, oxidation of NADH can induce this form of mPTP opening.([659])

(3) Preconditioning using calcium is believed to be mediated by the activation of kinases such as PKC.([372;375;378]) This form of mPTP opening may contribute to calcium signalling, through the phenomenon of mitochondrial Ca^{2+}-induced Ca^{2+} release (mCICR) via transitory opening of the mPTP.([647;648;815;837-839]) Therefore, the release of Ca^{2+} from mitochondria from this form of mPTP opening may activate kinases such as PKC; and

(4) Preconditioning has been demonstrated to protect the heart by uncoupling mitochondrial oxidative phosphorylation.([150]) Indeed modest mitochondrial uncoupling can mimic preconditioning-induced protection.([236;237]) Mitochondrial uncoupling can induce transient mPTP opening by either depolarising mitochondrial membrane potential,([906]) or by producing ROS.([907]) Conversely this form of mPTP opening would be expected to partially uncouple mitochondrial respiration.

(5) Garlid has suggested that the opening of the mitochondrial K_{ATP} channel induces matrix alkanisation.([110;132]) The K^+ influx induced by opening the mitochondrial K_{ATP} channel results in a proton efflux which results in matrix alkanisation.([110;132]) An increase in matrix pH would be expected to favour transient (low-conductance) mPTP opening since mPTP opening is inhibited by protons.([686])

On the basis of these common features we postulated that transient (low-conductance) mPTP opening prior to the index ischaemic period is required to mediate preconditioning-induced protection. In addition, we wanted to determine whether this form of mPTP opening contributed to the protection induced by modest mitochondrial uncoupling, a mechanism which can mimic preconditioning-induced protection. We were particularly interested in the role of ROS as a mediator of both preconditioning and mitochondrial uncoupling-induced protection and its relationship to transient (low-conductance) mPTP opening. Specifically, we postulated that this form of mPTP opening is required to mediate preconditioning and mitochondrial uncoupling-induced protection because it acts as a channel for mitochondrial ROS release.

7.2 HYPOTHESIS

Transient (low-conductance) opening of the mPTP mediates both preconditioning and mitochondrial uncoupling-induced protection by acting as a channel for mitochondrial ROS release.

In the first part of the study, our objective was to determine whether transient (low-conductance) mPTP opening is required to mediate both preconditioning and mitochondrial uncoupling-induced protection. Therefore, we subjected the isolated perfused rat heart to IPC, pharmacological preconditioning using diazoxide or CCPA (the adenosine A1-receptor agonist), or mitochondrial uncoupling using 2,4-dinitrophoenol (DNP). During the preconditioning protocols, we gave the known mPTP inhibitors cyclosporin-A([664]) or sanglifehrin-A,([676]) to inhibit mPTP opening, to determine whether preconditioning or mitochondrial uncoupling-induced protection would be abrogated.

In the second part of the study, our objective was to determine whether preconditioning actually induces transient (low-conductance) mPTP opening. We used a well-established model for detecting transient mPTP opening in the intact cell.([832]) In the final part of the study, we determined whether preconditioning-induced mitochondrial ROS release depends on mPTP opening, in order to establish whether transient (low-conductance) mPTP opening acts as a channel for mitochondrial ROS release into the cytosol. For this part of the study we used the fluorescent dye dichlorofluorescin (DCF) to detect mitochondrial ROS generation, which was assessed using flow cytometry.

7.3 Aim (1):

Determine whether pharmacologically inhibiting mPTP opening and scavenging ROS, during the preconditioning phase, abrogates preconditioning and/or mitochondrial-uncoupling induced protection.

7.3.1 Materials

Diazoxide (Dzx, Sigma Chemicals, Poole, Dorset), N-mercaptopropionylglycine (MPG, Sigma Chemicals, Poole, Dorset), sanglifehrin-A (SfA, Novartis Pharma AG, Basel), and 2,4-dinitrophoenol (DNP, Sigma Chemicals, Poole, Dorset), were dissolved in dimethyl sulfoxide (DMSO, Sigma Chemicals, Poole, Dorset) and added to the Krebs-Henseleit buffer such that the final DMSO concentration was less than 0.02%. Cyclosporin-A (CsA, Sigma Chemicals, Poole, Dorset) was dissolved in 50% ethanol and added to the Krebs-Henseleit buffer such that the final ethanol concentration was less than 0.005%. 2 chloro-N^6-cyclopentyl-adenosine (CCPA, Sigma Chemicals, Poole, Dorset) was dissolved in distilled water. All other reagents were of standard analytical grade.

7.3.2 Isolated Langendorff-Perfused Rat Heart Model

Hearts were excised from male Sprague-Dawley rats and mounted on a Langendorff-perfusion apparatus and subjected to 35 minutes regional ischaemia followed by 120 minutes of reperfusion (see section 3.2.1). At the end of the reperfusion period, the infarct-risk volume ratio was determined using tetrazolium (TTC) staining (see section 3.2.2).

7.3.3 Experimental Protocols for Infarct Studies

The experiment protocols for the infarct studies are presented in figure 7.1. The hearts were randomly assigned to one of the following treatment groups:

(1) ***Control hearts*** were perfused with 0.02% DMSO (n=6) prior to lethal ischaemia or 0.005% ethanol (n=6) or Krebs-Henseleit buffer alone (n=6) during stabilisation.

(2) ***IPC hearts*** (n=6) were treated with two 5 minute periods of global ischaemia with a 10-minute intervening reperfusion prior to the lethal ischaemic insult.

(3) ***CsA + IPC:*** Hearts (n=6) underwent IPC in the presence of the known mPTP inhibitor, cyclosporin-A (0.2 μmol/l).([664]) This drug was perfused throughout the IPC protocol. This

concentration of cyclosporin-A has been previously used to inhibit mPTP opening in the isolated perfused rat heart.([780])

(4) ***SfA + IPC:*** Hearts (n=6) underwent IPC in the presence of the known mPTP inhibitor, sanglifehrin-A (1.0 μmol/l).([676]) This drug was perfused throughout the IPC protocol. This concentration of sanglifehrin-A has been previously used to inhibit mPTP opening in the isolated perfused rat heart.([676])

(5) ***MPG + IPC:*** Hearts (n=6) underwent IPC in the presence of the free radical scavenger, MPG (1 mmol/l). MPG was perfused throughout the IPC protocol. This concentration of MPG has been previously used to scavenge free radicals in the isolated perfused rat heart.([908])

(6) ***Dzx treatment:*** Hearts (n=6) were perfused with diazoxide (30 μmol/l) for 10 minutes followed by 10 minutes wash-out with Krebs-Henseleit buffer prior to the lethal ischaemic insult. This concentration of diazoxide has been previously shown to precondition the isolated perfused rat heart.([104])

(7) ***CsA + Dzx:*** Hearts (n=6) were preconditioned with diazoxide in the presence of cyclosporin-A.

(8) ***SfA + Dzx:*** Hearts (n=6) were preconditioned with diazoxide in the presence of sanglifehrin-A.

(9) ***MPG + Dzx:*** Hearts (n=6) were preconditioned with diazoxide in the presence of MPG.

(10) ***CCPA treatment:*** Hearts (n=6) were perfused with the adenosine A1-receptor agonist, CCPA (200 nmol/l) for 10 minutes (during which time the hearts were paced at 300 beats per minute due to CCPA-induced bradycardia) followed by 10 minutes wash-out with Krebs-Henseleit buffer prior to the lethal ischaemic insult. This concentration of CCPA has been previously shown to precondition the isolated perfused rat heart.([880])

(11) ***CsA + CCPA:*** Hearts (n=6) were preconditioned with CCPA in the presence of cyclosporin-A.

(12) ***SfA + CCPA:*** Hearts (n=6) were preconditioned with CCPA in the presence of sanglifehrin-A.

(13) ***MPG + CCPA:*** Hearts (n=6) were preconditioned with CCPA in the presence of MPG.

(14) ***DNP treatment:*** Hearts (n=9) were perfused with 2,4-dinitrophoenol (50 nmol/l) for 5 minutes followed by 10 minutes wash-out with Krebs-Henseleit buffer prior to the lethal ischaemic insult. This concentration of DNP has been previously shown to precondition the isolated perfused rat heart.([236])

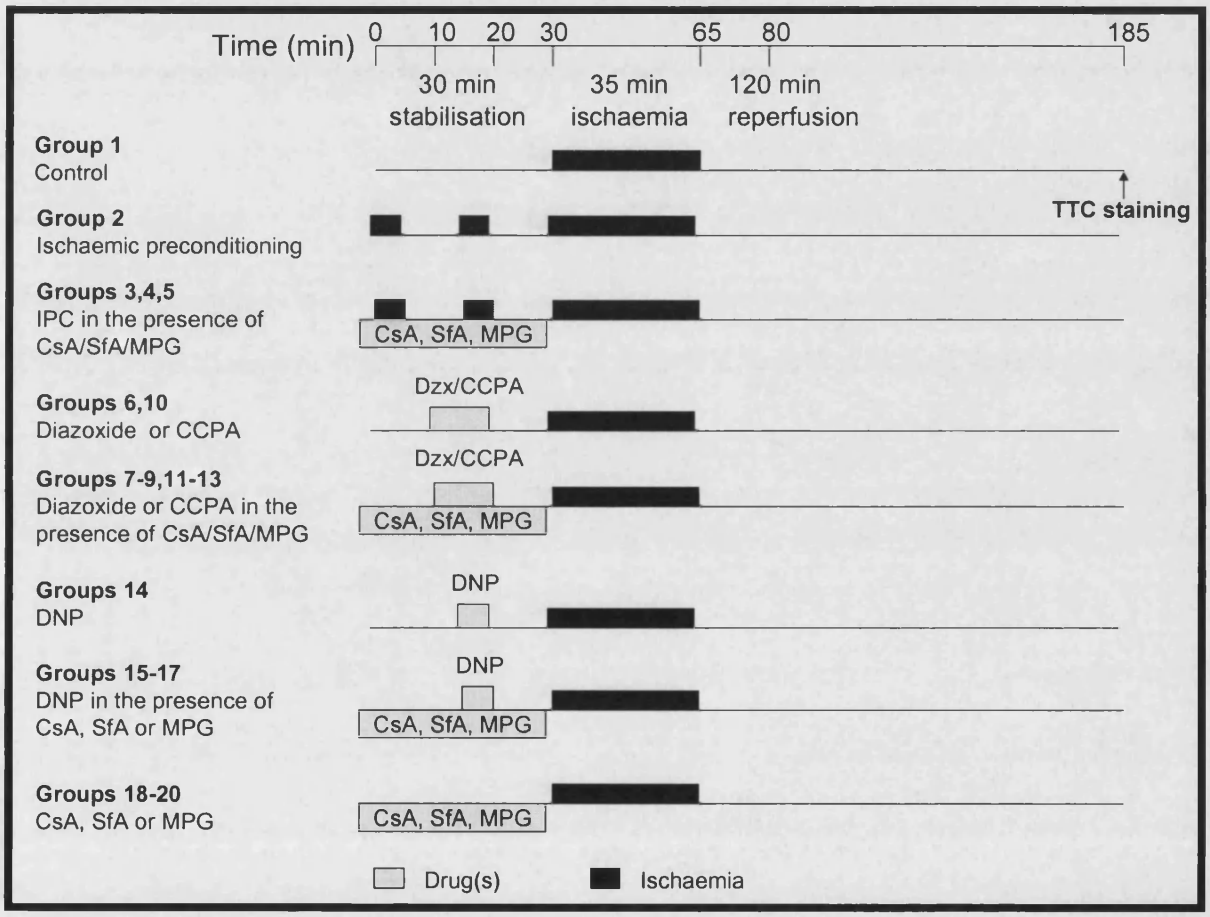
(15) ***CsA + DNP:*** Hearts (n=6) were perfused with DNP in the presence of CsA.

(16) ***SfA + DNP:*** Hearts (n=6) were perfused with DNP in the presence of SfA.

(17) ***MPG + DNP:*** Hearts (n=6) were perfused with DNP in the presence of MPG.

(18-20) Hearts (n=6 per group) were perfused with either CsA, SfA or MPG throughout stabilisation.

Figure 7.1: Experimental Protocols for Investigating the Role of the mPTP as a Mediator of Preconditioning-Induced Protection. TTC, triphenyltetrazolium chloride. IPC, ischaemic preconditioning. CsA, cyclosporin-A. SfA, sanglifehrin-A. MPG, 3-mercaptopropionylglycine. Dzx, diazoxide. 2 chloro-N^6-cyclopentyl-adenosine , CCPA. DNP, 2,4-dinitrophoenol.



7.3.4 Results

7.3.4.1 Exclusions

We used 158 male Sprague-Dawley rat hearts for the infarct size experiments of which 11 were excluded owing to poor function during stabilisation (see section 3.2.3 for exclusion criteria).

7.3.4.2 Animal and Haemodynamic Data

Animal and heart weights and risk zone volumes were similar in the experimental groups (see table 7.1). Baseline data relating to cardiac function (assessed by the rate-pressure-product, RPP) and coronary flow before the lethal ischaemic period, were similar in the experimental groups (see table 7.2, 7.3). During regional ischaemia, coronary flow and RPP decreased to a similar extent in both groups. An increase in cardiac function and coronary flow upon reperfusion was indicative of successful re-flow.

Table 7.1 Characteristics of Animals in Treatment Groups

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
1. Control	12	363±12	1.86±0.10	0.527±0.021
DMSO	3	360±9	1.98±0.05	0.614±0.053
Ethanol	3	365±4	1.83±0.08	0.538±0.014
2. IPC	10	330±11	1.87±0.08	0.481±0.024
3. CsA + IPC	6	337±10	1.84±0.13	0.473±0.044
4. SfA + IPC	6	337±3	1.81±0.04	0.525±0.035
5. MPG + IPC	6	302±13	1.64±0.07	0.449±0.032
6. Dzx	6	381±12	1.95±0.05	0.543±0.033
7. CsA + Dzx	6	374±10	1.87±0.06	0.516±0.026
8. SfA + Dzx	7	421±16	2.10±0.08	0.543±0.027
9. MPG + Dzx	6	349±8	1.60±0.05	0.522±0.027
10. CCPA	9	384±7	1.94±0.09	0.495±0.021
11. CsA + CCPA	6	409±11	1.90±0.08	0.506±0.034
12. SfA + CCPA	6	388±19	2.22±0.12	0.548±0.036
13. MPG + CCPA	6	361±5	1.68±0.07	0.513±0.025
14. DNP	11	397±22	2.00±0.09	0.508±0.023
15. CsA + DNP	7	427±29	2.09±0.12	0.487±0.039
16. SfA + DNP	6	463±27	2.30±0.17	0.512±0.044
17. MPG + DNP	6	354±27	1.89±0.18	0.416±0.028
18. CsA	6	425±38	2.16±0.17	0.534±0.028
19. SfA	6	389±29	2.07±0.13	0.515±0.036
20. MPG	7	372±16	1.77±0.11	0.436±0.033

Values are mean±SEM. *P<0.001 compared with control.

Table 7.2 Rate Pressure Product (x 10^3mmHg/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	28.7±3.0	27.5±2.5	11.9±1.7	20.8±1.5	23.2±2.5	14.8±1.7
DMSO	30.8±2.2	28.6±3.0	7.2±1.1	20.7±1.9	21.1±1.2	16.8±2.8
Ethanol	36.1±8.6	28.6±7.0	8.8±2.1	18.4±1.9	25.5±3.1	19.2±2.0
2. IPC	26.7±3.5	23.1±2.4	13.4±2.7	19.7±2.0	25.4±2.7	18.3±2.5
3. CsA + IPC	24.3±2.6	24.1±3.7	10.8±3.0	12.8±1.4	17.6±2.6	12.3±3.0
4. SfA + IPC	26.5±9.2	24.0±6.8	12.9±1.8	22.1±8.1	21.0±1.8	11.4±3.2
5. MPG + IPC	39.8±2.4	34.1±2.5	14.9±2.2	20.5±1.3	20.9±2.9	14.5±1.4
6. Dzx	26.3±3.0	27.3±3.1	13.1±3.2	19.6±3.0	30.3±1.4	21.3±2.9
7. CsA + Dzx	29.7±4.2	33.7±3.8	15.3±2.7	22.0±2.2	27.3±2.3	18.0±1.6
8. SfA + Dzx	29.5±5.7	27.5±2.0	11.3±2.5	18.7±1.9	26.7±1.2	16.0±1.6
9. MPG + Dzx	29.7±2.7	37.2±2.4*	16.8±1.7	20.7±2.1	17.5±3.3	10.1±2.8
10.CCPA	30.5±1.5	22.6±2.3	9.4±1.4	19.8±2.3	21.1±2.0	11.2±1.1
11.CsA+CCPA	48.3±2.9*	26.3±6.2	10.9±0.9	23.6±1.2	24.3±3.3	13.2±2.5
12.SfA+ CCPA	47.3±4.1*	22.1±4.5	7.9±1.4	22.0±3.6	22.1±3.5	12.5±1.8
13.MPG +CCPA	36.2±4.6	35.0±4.4	20.1±1.8	22.9±1.1	32.3±3.2	18.7±1.2
14.DNP	27.4±2.4	23.6±2.0	12.3±1.3	20.4±1.3	26.1±1.9	19.1±1.4
15.CsA + DNP	26.6±3.3	22.4±2.5	9.2±1.3	18.8±2.0	22.5±1.9	16.2±1.4
16.SfA + DNP	30.7±2.8	16.5±2.3*	10.8±1.9	21.6±1.1	23.1±2.9	18.8±2.2
17.MPG + DNP	26.0±5.2	27.5±2.1	17.9±3.8	21.4±2.0	20.1±1.4	9.5±2.0
18.CsA	27.5±4.7	33.4±2.0	10.4±1.4	19.6±2.3	26.4±1.5	19.1±0.7
19.SfA	24.4±2.3	31.6±3.5	10.7±1.8	18.4±1.8	22.8±2.1	14.8±3.6
20. MPG	29.2±2.9	32.5±3.3	16.5±2.2	16.4±2.8	32.4±3.2	18.7±1.2

Values are mean±SEM. *P<0.05 compared with control.

Table 7.3 Coronary Flow Rate (ml/min)

Groups	Stabilisation		Ischaemia		Reperfusion	
	5-Min	30-Min	5-Min	30-Min	15-Min	120-Min
1. Control	17.8±1.3	17.7±1.5	10.8±1.7	11.9±1.8	16.9±1.8	12.4±2.0
DMSO	17.0±1.0	15.3±1.2	8.0±0.6	9.7±0.3	14.7±1.3	9.7±0.3
Ethanol	18.0±1.2	17.3±1.2	7.7±0.3	8.7±0.9	16.0±0.6	10.0±0.6
2. IPC	14.7±1.0	16.2±0.8	7.4±0.6	7.7±0.7	14.2±0.6	9.3±0.7
3. CsA + IPC	21.7±2.0	19.0±3.1	6.5±0.3	7.6±1.0	12.6±1.4	7.5±0.6
4. SfA + IPC	24.0±1.0*	24.0±2.3*	11.5±2.4	19.9±0.1	24.0±2.0	12.0±2.5
5. MPG + IPC	17.0±1.6	19.2±1.7	9.7±1.1	9.5±0.9	17.2±1.5	10.7±1.6
6. Dzx	18.8±1.6	18.0±1.8	8.8±0.9	10.3±1.4	17.8±2.7	12.8±1.9
7. CsA + Dzx	16.3±1.0	18.3±1.7	8.7±0.7	9.0±0.8	14.7±1.3	9.0±0.9
8. SfA + Dzx	18.4±1.8	17.0±1.4	8.0±0.6	9.0±0.6	16.5±0.7	10.0±0.6
9. MPG + Dzx	21.4±1.1	22.5±1.3*	11.5±0.6	12.5±1.6	20.0±2.1	11.7±1.9
10.CCPA	21.0±0.7	20.4±1.2	9.8±0.4	11.2±0.8	15.8±0.5	10.0±0.5
11.CsA+CCPA	22.3±2.0*	20.0±0.8	8.8±0.7	10.0±0.9	17.3±0.7	10.8±1.2
12.SfA+ CCPA	23.0±1.0*	22.3±1.7*	9.4±1.5	11.2±1.4	19.3±0.8	11.8±1.5
13.MPG +CCPA	25.0±1.7*	26.0±0.9*	11.8±0.6	13.2±0.4	21.8±1.5	14.7±1.8
14.DNP	19.6±1.5	19.5±1.1	8.9±0.3	10.9±0.6	19.7±0.9	12.8±0.8
15.CsA + DNP	18.5±1.3	17.6±1.0	8.9±0.7	10.8±1.0	17.2±1.2	11.9±1.4
16.SfA + DNP	19.0±0.9	19.2±1.4	9.2±0.4	11.0±0.8	16.8±0.8	10.3±0.7
17.MPG + DNP	17.7±3.2	19.3±1.8	10.0±0.6	11.7±0.9	18.0±1.7	10.0±1.0
18.CsA	19.3±2.3	20.0±2.2	8.7±0.7	10.2±1.0	18.5±2.5	13.0±1.9
19.SfA	16.3±1.1	16.3±0.9	8.5±1.1	9.7±1.2	14.8±0.7	9.3±0.8
20. MPG	20.9±1.9	20.4±1.2	11.1±0.9	9.1±0.7	15.9±1.1	8.6±0.7

Values are mean±SEM. *P<0.05 compared with control.

7.3.4.3 Infarct Size Data

Inhibiting mPTP Opening Abrogates Preconditioning-Induced Protection

Ischaemic preconditioning (IPC) reduced infarct size from 49.9±3.8% in control to 20.2±3.6% with IPC (P<0.001; figure 7.2a). Inhibiting mPTP opening during the preconditioning protocol, using either of the known mPTP inhibitors CsA or SfA, abolished the protection associated with IPC (20.2±3.6% vs 45.9±2.5% with CsA, 49.0±7.1% with SfA; P<0.001; figure 7.2a), suggesting that mPTP opening is required to mediate the protection in this setting.

Pharmacological preconditioning using either diazoxide or CCPA reduced infarct size from 49.9±3.8% in control to 22.1±2.7% with diazoxide, 24.9±4.5% with CCPA (P<0.001; figures 7.2b, 7.3a). Inhibiting mPTP opening during the preconditioning protocol, using either of the known mPTP inhibitors CsA or SfA, abolished the protection associated with diazoxide (22.1±2.7% vs 46.3±3.0% with CsA, 48.4±5.5% with SfA; P<0.001; figure 7.2a) and CCPA (24.9±4.5% vs 54.4±6.6% with CsA, 42.6±9.0% with SfA; P<0.001; figure 7.3a), suggesting that mPTP opening is required to mediate the protection in this setting.

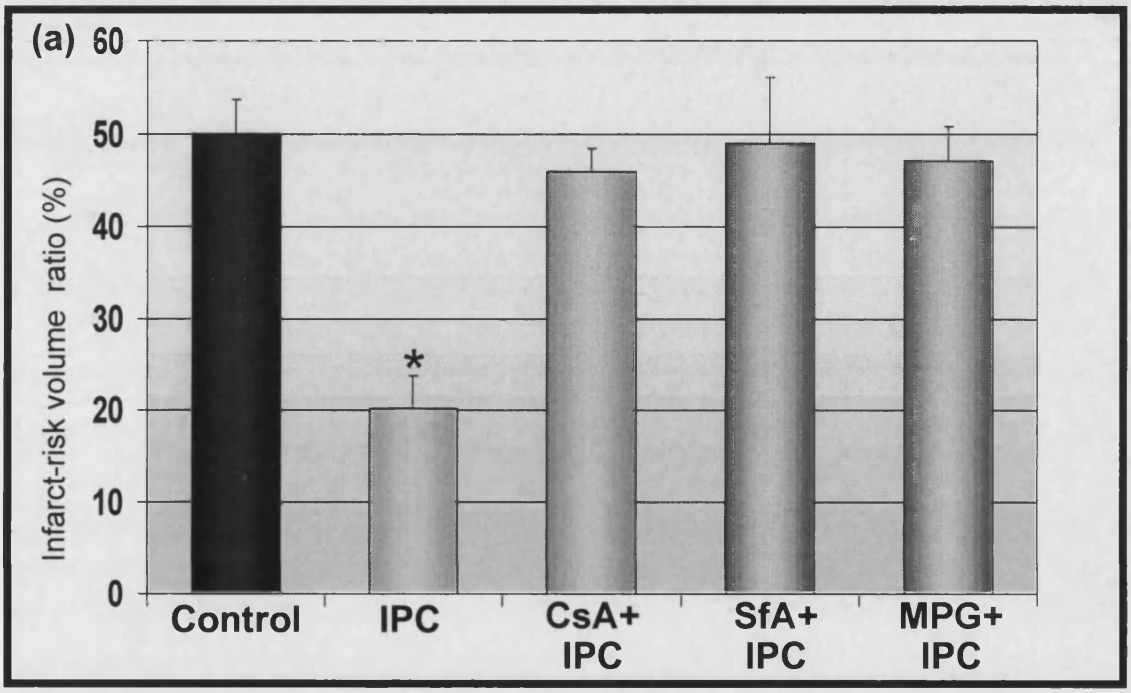
Treatment with the mitochondrial-uncoupler, DNP reduced infarct size from 49.9±3.8% in control to 15.7±2.7% with DNP (P<0.001; figure 7.3b). Inhibiting mPTP opening during the preconditioning protocol, using either of the known mPTP inhibitors CsA or SfA, also abolished the protection associated with DNP (15.7±2.7% vs 40.8±5.5% with CsA, 34.3±3.1% with SfA; P<0.001; figure 7.3b), suggesting that mPTP opening is required to mediate the protection in this setting.

Scavenging Reactive Oxygen Species and Preconditioning-Induced Protection

The presence of the ROS scavenger, MPG, during the preconditioning protocols abolished the protection associated with IPC (20.2±3.6% vs 47.1±3.8% with MPG; P<0.001; figure 7.2a), diazoxide (22.1±2.7% vs 56.3±3.8% with MPG; P<0.001; figure 7.2b), implicating ROS as a mediator of protection in these settings. However, MPG did not abolish the protection associated with CCPA (24.9±4.5% vs 26.5±8.4% with MPG; P<0.001; figure 7.3a). The presence of the ROS scavenger, MPG, during the preconditioning protocols abolished the protection associated with DNP (15.7±2.7% vs 50.7±6.6% with MPG; P<0.001; figure 7.3b)

Given alone during stabilisation, neither cyclosporin-A, sanglifehrin-A nor MPG influenced infarct size (43.9±1.4% in control vs 42.8±3.5% with CsA, 48.0±4.2% with SfA, 47.8±6.4% with MPG; P=NS; figure 7.4).

Figure 7.2 (a-b): Inhibiting mPTP Opening and Scavenging ROS Abrogates Preconditioning-Induced Protection: Inhibiting mitochondrial permeability transition pore (mPTP) opening using either cyclosporin-A (CsA) or sanglifehrin-A (SfA) or scavenging ROS using 3-mercaptopropionylglycine (MPG) during the preconditioning phase abolishes the protection associated with (a) ischaemic preconditioning (IPC) and (b) diazoxide (Dzx). Values are Mean±SEM.*P<0.01.



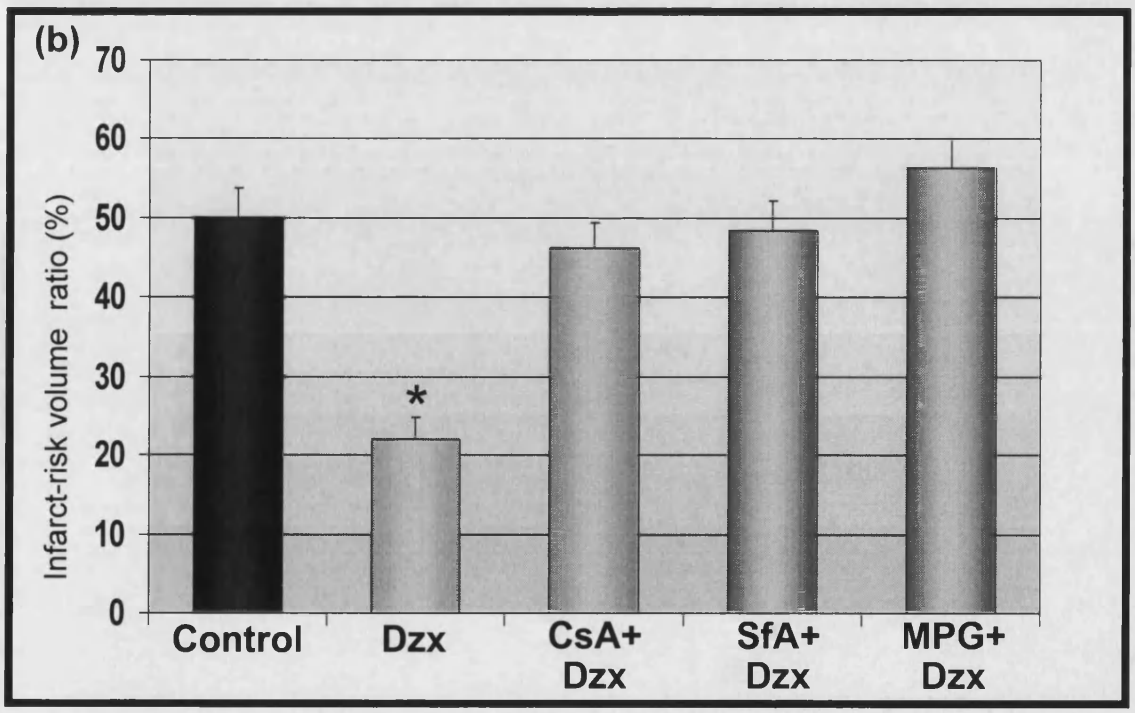
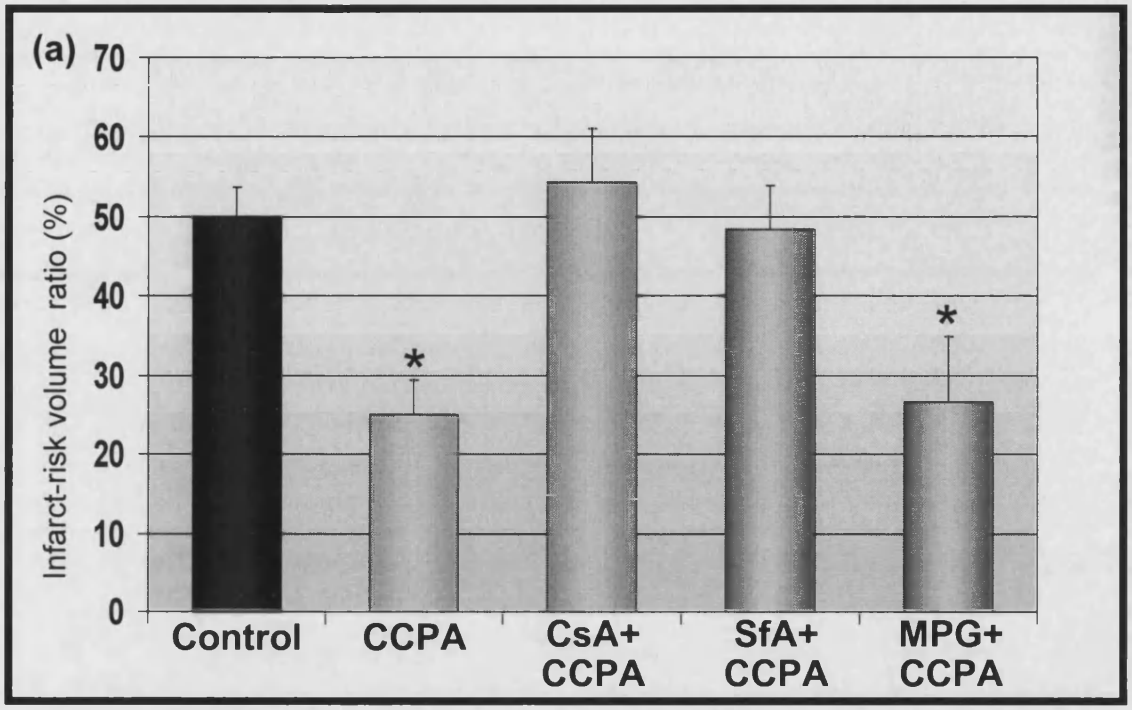


Figure 7.3 (a-b): Inhibiting mPTP Opening and Scavenging ROS Abrogates Preconditioning-Induced Protection: Inhibiting mitochondrial permeability transition pore (mPTP) opening using either cyclosporin-A (CsA) or sanglifehrin-A (SfA) during the preconditioning phase abolishes the protection associated with (a) 2 chloro-N^6-cyclopentyl-adenosine (CCPA) and (b) 2,4-dinitrophoenol (DNP). However, scavenging ROS using 3-mercaptopropionylglycine (MPG) abolishes the protection associated with DNP but not CCPA. Values are Mean±SEM.*P<0.01.



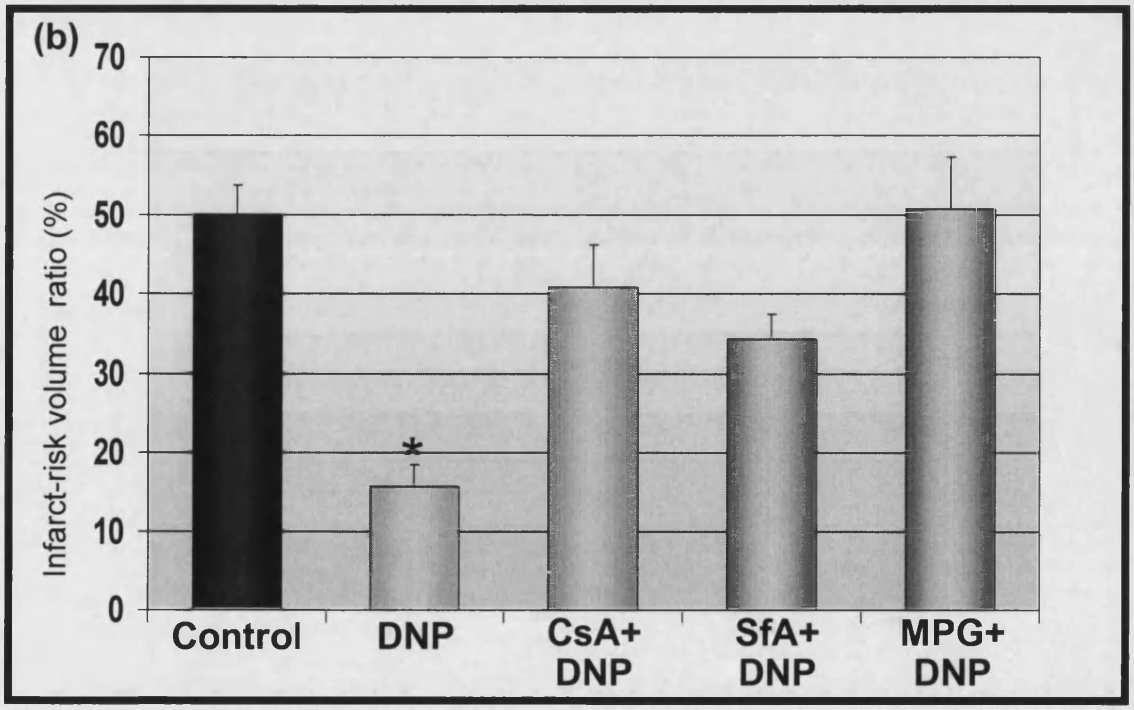
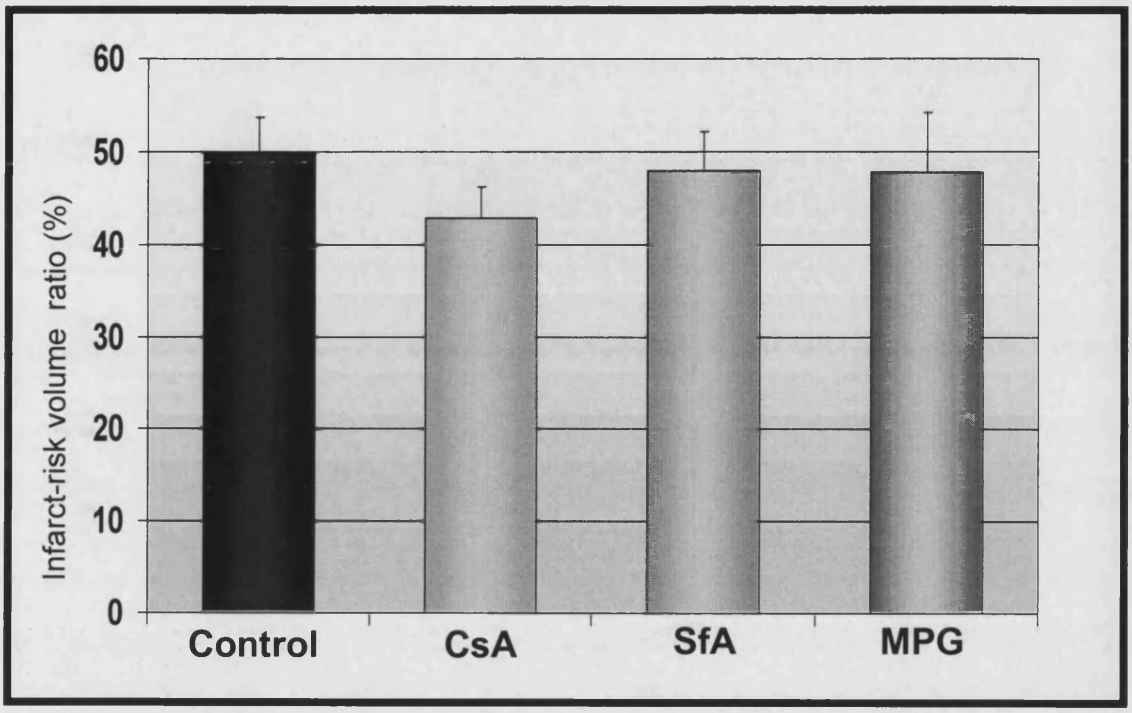


Figure 7.4: The Effect of CsA, SfA and MPG Alone: The presence of either cyclosporin-A (CsA), sanglifehrin-A (SfA) or 3-mercaptopropionylglycine (MPG) during stabilisation did not influence infarct size. Values are Mean±SEM.*P<0.01.



7.3.5 Discussion

In this part of the study, we demonstrated for the first time that transient (low-conductance) opening of the mPTP during the preconditioning phase, is required to mediate the protection associated with both ischaemic and pharmacological preconditioning and mitochondrial-uncoupling. We demonstrated that pharmacologically inhibiting mPTP opening during the preconditioning phase, completely abrogated the protection associated with IPC, diazoxide, and CCPA, indicating that mPTP opening is required for protection in these settings. This suggests that preconditioning using these different approaches induces mPTP opening during the preconditioning phase. The possible mechanisms which may explain how preconditioning-induces transient (low-conductance) mPTP opening are discussed in section 7.4.5.

We also showed for the first time that mitochondrial-uncoupling induced protection is mediated by ROS, as evidenced by the abrogation of protection in this setting in the presence of the free radical scavenger, MPG. We confirm that IPC and diazoxide-induced protection is ROS-dependent,([67;72;116]) and demonstrate that CCPA-induced preconditioning is ROS-independent, supporting the findings of Cohen and colleagues.([73])

7.3.5.1 The Role of the mPTP and ROS in Mitochondrial Uncoupling-Induced Protection

It is has been previously demonstrated that ischaemic preconditioning and diazoxide induce mitochondrial uncoupling.([108;150;257]) Furthermore, modest mitochondrial uncoupling can mimic preconditioning-induced protection.([236;237]) The mechanism by which mitochondrial uncoupling protects the heart against ischaemia-reperfusion injury is not clear but it may in part be due to an attenuation in mitochondrial Ca^{2+} load.([52;108;258]) In the present study, we confirm that modest mitochondrial uncoupling can mimic preconditioning-induced protection as evidenced by a reduction in infarct size with pre-treatment with dinitrophoenol. We show for the first time that mitochondrial uncoupling-induced protection is mediated by both mPTP opening and ROS, suggesting that both transient (low-conductance) mPTP opening and ROS occur as a consequence of mitochondrial-uncoupling. Mitochondrial uncoupling can induce transient (low-conductance) mPTP opening by mitochondrial membrane depolarisation.([906]) Vercesi's group have also demonstrated that mitochondrial-uncoupling induces the production of mitochondrial hydrogen peroxide, which then induced mPTP opening.([907]) This finding may explain why we observed that the protection induced by mitochondrial-uncoupling to be dependent on ROS.

Therefore, we can postulate that mitochondrial-uncoupling mimics preconditioning-induced protection by generating mitochondrial ROS, which then activate kinases implicated in preconditioning-induced protection.

7.3.5.2 The Mechanism by Which Transient mPTP Opening Mediates Protection

Transient (low-conductance) mPTP opening during the preconditioning phase, induced by the preconditioning stimulus or mitochondrial uncoupling, may mediate protection by: (1) Reducing mitochondrial Ca^{2+} load.([647]) In this regard, Katoh and colleagues have demonstrated that diazoxide at a very high concentration (200 µmol/l) can induce mitochondrial calcium-efflux via mPTP opening.([266]) Interestingly, in an earlier study, Holmuhamedov and colleagues,([257;258]) demonstrated using isolated mitochondria that diazoxide at a very high concentration (IC_{50} 96 µmol/l) induced both mitochondrial Ca^{2+} and cytochrome C release, which were both sensitive to cyclosporin-A, suggesting the involvement of mPTP opening; (2) Mediating mitochondrial ROS release/signalling.([840]) Whether preconditioning-induced mitochondrial ROS release occurs via mPTP opening was investigated in section 7.5; (3) Facilitating Ca^{2+} signalling and subsequent kinase activation. Preconditioning using calcium has been demonstrated to be mediated by the activation of kinases such as PKC.([372;375;378]) One might expect the transient intracellular increase in Ca^{2+} to induce transient (low-conductance) mPTP opening which in turn would cause mitochondrial Ca^{2+} efflux. But whether, the released Ca^{2+} activates kinases implicated in preconditioning is unknown.

To implicate mPTP opening as a mediator of protection it was essential to investigate the effects of two different mPTP inhibitors. SfA has the advantage over CsA in that it does not inhibit the protein phosphatase, calcineurin.([677;678]) However, the infarct studies do not demonstrate directly that transient (low-conductance) mPTP opening actually occurs in the setting of preconditioning. Its occurrence is only implicated with the use of pharmacological inhibitors, which may be sufficient to infer the involvement of the mPTP. To demonstrate directly, that preconditioning induces transient (low-conductance) mPTP opening, we investigated the effect of diazoxide on mPTP opening in myocytes.

7.4 Aim (2):

Determine whether myocardial preconditioning causes transient (low-conductance) opening of the mPTP

In order to determine whether preconditioning induces transient (low-conductance) mPTP opening during the preconditioning phase, we investigated the effect of the preconditioning mimetic, diazoxide on mPTP opening in adult rat myocytes, using a model which was devised by Di Lisa's group for detecting transient (low-conductance) mPTP opening.([832])

7.4.1 Materials

Diazoxide was dissolved in dimethyl sulphoxide (DMSO, Sigma Chemicals, Poole, Dorset), giving a final concentration of <0.1% DMSO. Cyclosporin-A (Sigma Chemicals, Poole, Dorset) was dissolved in 50% ethanol, giving a final concentration of 0.05% ethanol. 5-Hydroxydecanoic acid (5-HD, Sigma Chemicals, Poole, Dorset) was dissolved in distilled H_2O. The fluorescent dye, calcein-AM (calcein-acetoxymethyl ester, Molecular Probes Inc., Leiden, The Netherlands) was dissolved in dimethyl sulphoxide (DMSO). All other reagents were of standard analytical grade.

7.4.2 Myocyte Model of Transient (Low-Conductance) mPTP Opening

Adult rat myocytes were isolated from male Sprague-Dawley rats according to the method described in section 3.5. Isolated myocytes were seeded onto 25-mm round cover-slips according to the method described in section 3.6.

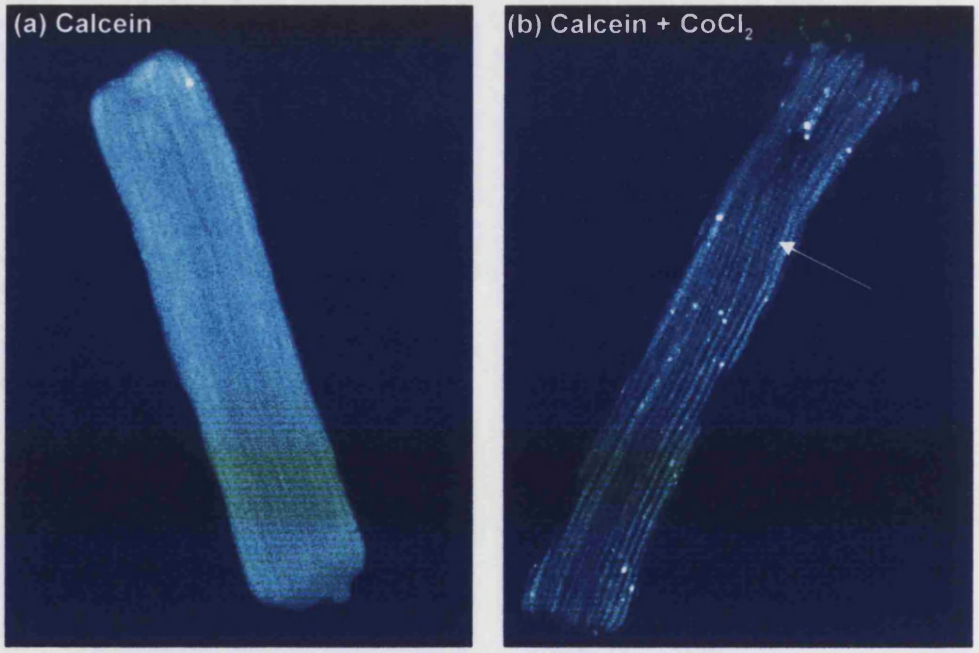
For this part of the study, a well-established reproducible method for detecting transient (low-conductance) opening of the mPTP in the intact cell was used.([266;832]) Petronilli and colleagues observed that under steady state conditions there was a slow loss of mitochondrial calcein, which was inhibited by CsA, suggesting transient mPTP opening under physiological conditions.([832]) Using this model we examined whether diazoxide (at a cardio-protective concentration)([104]) induced transient mPTP opening.

Myocytes were incubated with the fluorescent dye calcein-AM (1 μmol/l) and cobalt chloride ($CoCl_2$, 1 mmol/l) for 30 minutes at 37°C. Intracellular esterases remove the acetoxymethyl ester group, releasing free calcein which because it is membrane-impermeable,

becomes trapped within the cytosol and mitochondria. The cytosolic calcein signal is then quenched by the $CoCl_2$, leaving the calcein signal localised to the mitochondria, which can then be visualised by confocal microscopy (see figure 7.5b). Figure 7.5a depicts a representative myocyte loaded with calcein-AM in the absence of $CoCl_2$ and demonstrates homogenous calcein fluorescence, with localisation of mitochondria impossible. With the calcein trapped within the mitochondrial matrix, the calcein can only exit if the mPTP opens. Therefore, in this model, transient (low-conductance) mPTP opening is represented by a reduction in mitochondrial calcein fluorescence, which can be measured using confocal microscopy.

Mitochondrial calcein fluorescence intensity was measured over six randomly chosen regions of interest (ROI's) in 3 different cells every 5 minutes for a total of 25 minutes. The mean mitochondrial calcein fluorescence intensity was expressed as the percentage of the baseline value.

Figure 7.5: Myocyte Model of mPTP Opening. Representative confocal image of adult rat myocyte loaded with calcein-AM alone (a) or with both calcein and cobalt chloride (b). In the absence of cobalt chloride, the myocyte displays an homogenous calcein fluorescence. However, in the presence of cobalt chloride, which quenches the cytosolic and nuclear calcein fluorescence signal, the calcein localises to the mitochondria, and appears as green stripes (see arrow). The trapped calcein within the mitochondria can only exit if the mPTP opens. Therefore, opening of the mPTP is indicated by a reduction in mitochondrial calcein fluorescence.



7.4.3 Experimental Protocols for Myocyte Studies

After incubating with calcein-AM/$CoCl_2$ in the restoration buffer, cells were randomly assigned to the following treatment groups:

(1) Control (n=18), or incubation with 0.05% ethanol vehicle (n=6), or 0.1% DMSO vehicle (n=6).

(2) Diazoxide (30 μmol/l, n=18).

(3) Diazoxide and CsA (0.2 μmol/l, a known inhibitor of the mPTP, n=18).

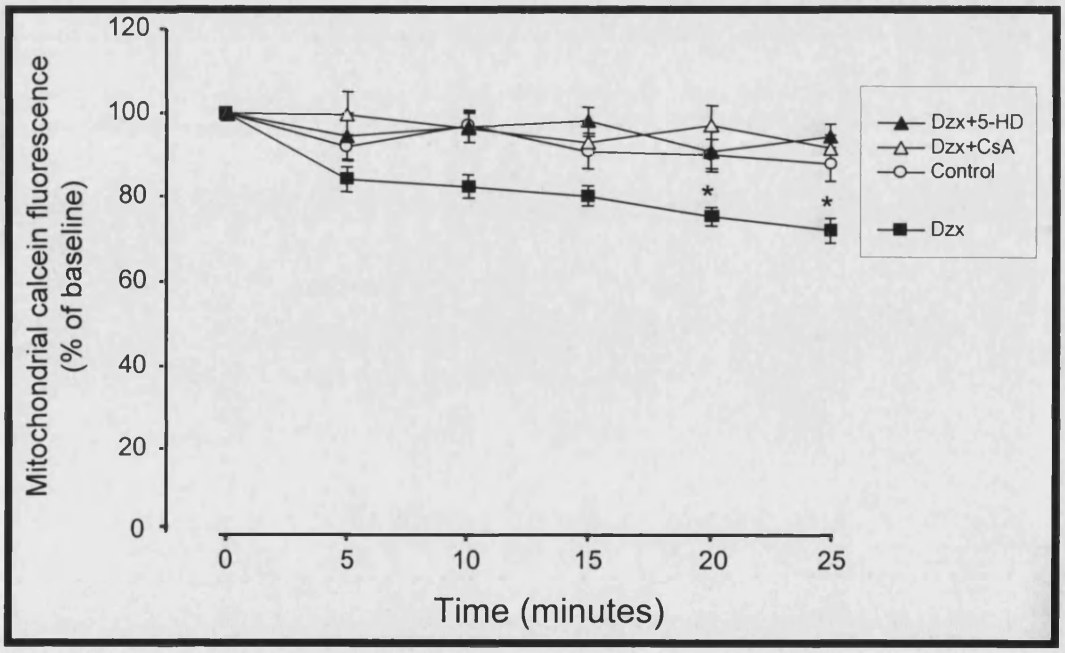
(4) Diazoxide and 5-HD (100 μmol/l, a purported mitochondrial K_{ATP} channel blocker, n=18).

(5) CsA (n=18), and (6) 5-HD (n=18).

7.4.4 Results

Treatment with diazoxide was shown to induce transient opening of the mPTP as shown by a reduction in mitochondrial calcein fluorescence to 72±3% of baseline values (figure 7.6). Importantly, mPTP opening did not result in cell death suggesting that it was of the transient non-lethal type. Diazoxide-induced transient opening of the mPTP was abrogated in the presence of cyclosporin-A (the known mPTP inhibitor), confirming that the reduction in mitochondrial calcein fluorescence actually indicates opening of the mPTP. Furthermore, diazoxide-induced transient opening of the mPTP was also abolished in the presence of the purported mitochondrial K_{ATP} channel blocker, 5-HD.

Figure 7.6: The Effect of Diazoxide on Transient (Low-Conductance) mPTP Opening. Change in mitochondrial calcein fluorescence (expressed as a percentage of the baseline fluorescence), demonstrating a diazoxide (Dzx)-induced reduction in mitochondrial calcein fluorescence, representing transient (low-conductance) opening of the mPTP, which is abolished in the presence of either 5-hydroxydecanoate (5-HD, a mitochondrial K_{ATP} channel blocker) or cyclosporine-A (CsA, an inhibitor of mPTP opening). Values are Mean±SEM. N= 36 per group. *P<0.05.



7.4.5 Discussion

In this section of the study, we demonstrated that the preconditioning-mimetic, diazoxide can induce transient (low-conductance) mPTP opening in myocytes under steady state conditions. The reduction in mitochondrial calcein fluorescence was used to indicate diazoxide-induced mPTP opening, and was demonstrated to be sensitive to the archetypal mPTP inhibitor CsA, confirming that the reduction in mitochondrial calcein fluorescence was actually due to mPTP opening. In addition, the effect of diazoxide on mPTP opening was blocked by 5-HD, the putative mitochondrial K_{ATP} channel blocker, suggesting that the effect of diazoxide on mPTP opening may be dependent on the mitochondrial K_{ATP} channel.

The mechanism by which diazoxide induces transient (low-conductance) mPTP opening is unknown but possible mechanisms include: (1) As mitochondrial K_{ATP} channel opening induces matrix alkanisation,([110;132]) and an increase in matrix pH would be expected to favour mPTP opening,([686]) this may be the potential mechanism by which diazoxide-induces transient (low-conductance) mPTP opening; (2) Oxidation of NADH has been demonstrated to induce transient (low-conductance) mPTP opening in isolated liver mitochondria,([659]) and diazoxide has been shown to generate mitochondrial ROS.([72;116]) Therefore, diazoxide-induced ROS may induce transient (low-conductance) mPTP opening by oxidising NADH; (3) diazoxide has been demonstrated to induce mitochondrial uncoupling.([108;150;257;846]) Mitochondrial uncoupling by either inducing mitochondrial membrane depolarisation([906]) or via ROS release([907]) may cause transient (low-conductance) mPTP opening.

The question remains as to how transient (low-conductance) mPTP opening mediates the protection associated with preconditioning and mitochondrial uncoupling. We have demonstrated that both forms of protection are dependent on ROS (except for CCPA-induced preconditioning). In the next section, we examined whether transient (low-conductance) mPTP opening is required to mediate the preconditioning-induced ROS release from mitochondria.

7.5 Aim (3)

Determine whether transient (low-conductance) opening of the mPTP acts as a channel for the release of ROS generated in response to preconditioning

In order to investigate a possible mechanism to explain why transient (low-conductance) opening of the mPTP is required for preconditioning-induced protection, this section of the study examines the role of this form of mPTP opening in mediating the mitochondrial ROS release that occurs in response to a preconditioning stimulus. Certain types of mitochondrial generated ROS such as superoxide are membrane impermeable and therefore require a channel to enter the cytosol.([905]) Marban's([862]) and Vanden Hoek's([49]) groups have suggested that the inner membrane anion channel (IMAC) may act as a channel for mitochondrial ROS release into the cytosol.

Because we have demonstrated that both ROS and transient (low-conductance) opening of the mPTP are required for preconditioning-induced protection and Zorov and colleagues([840]) have demonstrated that ROS generated within the mitochondrial matrix can induce the mitochondrial release of ROS via transient (low-conductance) opening of the mPTP, we postulated that the mPTP acts as a channel for the release of ROS generated in response to a preconditioning stimulus.

In order to investigate this proposition we determined whether inhibiting mPTP opening using CsA could block the mitochondrial ROS release induced by the preconditioning mimetic diazoxide, using isolated adult rat mitochondria. These studies are preliminary investigations and require further confirmatory studies.

7.5.1 Materials

Diazoxide was dissolved in dimethyl sulphoxide (DMSO, Sigma Chemicals, Poole, Dorset), giving a final concentration of <0.1% DMSO. Cyclosporin-A (Sigma Chemicals, Poole, Dorset) was dissolved in 50% ethanol, giving a final concentration of 0.05% ethanol. 5-Hydroxydecanoic acid (5-HD, Sigma Chemicals, Poole, Dorset) and 2 chloro-N^6-cyclopentyl-adenosine (CCPA, Sigma Chemicals, Poole, Dorset), were dissolved in distilled H_2O. The fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes Inc., Leiden, The Netherlands) was

dissolved in dimethyl sulphoxide (DMSO, Sigma Chemicals, Poole, Dorset) such that the final DMSO concentration was less than 0.1%. All other reagents were of standard analytical grade.

7.5.2 Mitochondrial ROS Release

In this study, mitochondrial ROS release was detected using DCF fluorescence and was measured by flow cytometry. Mitochondria were isolated from male Sprague-Dawley rats according to the method described in section 3.3. Aliquots of the mitochondrial sample (0.5 mg/ml) were incubated with the fluorescent dye, DCFH-DA (10 μmol/L) for 15 minutes at 37°C. This concentration of DCFH-DA has been previously used to detect diazoxide-induced ROS release in adult rat myocytes.([116]) Once within the matrix, the acetate group in DCFH-DA is removed by esterases, resulting in a non-fluorescent product DCFH.([116]) Oxidation of DCFH by ROS generates the fluorescent product DCF, which can be measured by flow cytometry. The mitochondria were then washed twice with KCl buffer containing (in mmol/l) KCl 120.0, TES 5.0, and $MgCl_2$ 0.1, with added ATP 0.2 and sodium succinate 10.

7.5.3 Experimental Protocols for Mitochondrial Studies

DCF-loaded mitochondria in KCl buffer were incubated for 30 minutes at room temperature with the following treatments:

(1) ***Control***: DMSO 0.1%

(2) ***Dzx***: Diazoxide (30 μmol/l). This concentration of diazoxide has been demonstrated to be cardio-protective.([104])

(3) ***Dzx+CsA***: Diazoxide (30 μmol/l) was incubated with CsA (0.2 μmol/l). This concentration of CsA has been demonstrated to inhibit mPTP opening.([779;780])

(4) ***CsA***

Mitochondrial DCF fluorescence was determined using the flow cytometer (see section 3.4) before adding the treatment and was then re-measured 30 minutes later. For DCF-fluorescence, the signal was analysed in the FL1 detector channel equipped with a band-pass filter at 520 nm; the photo-multiplier value of the detector was 631 V. Experiments were performed on mitochondria isolated from 2 individual rats, and were repeated three times for each isolation.

7.5.4 Results

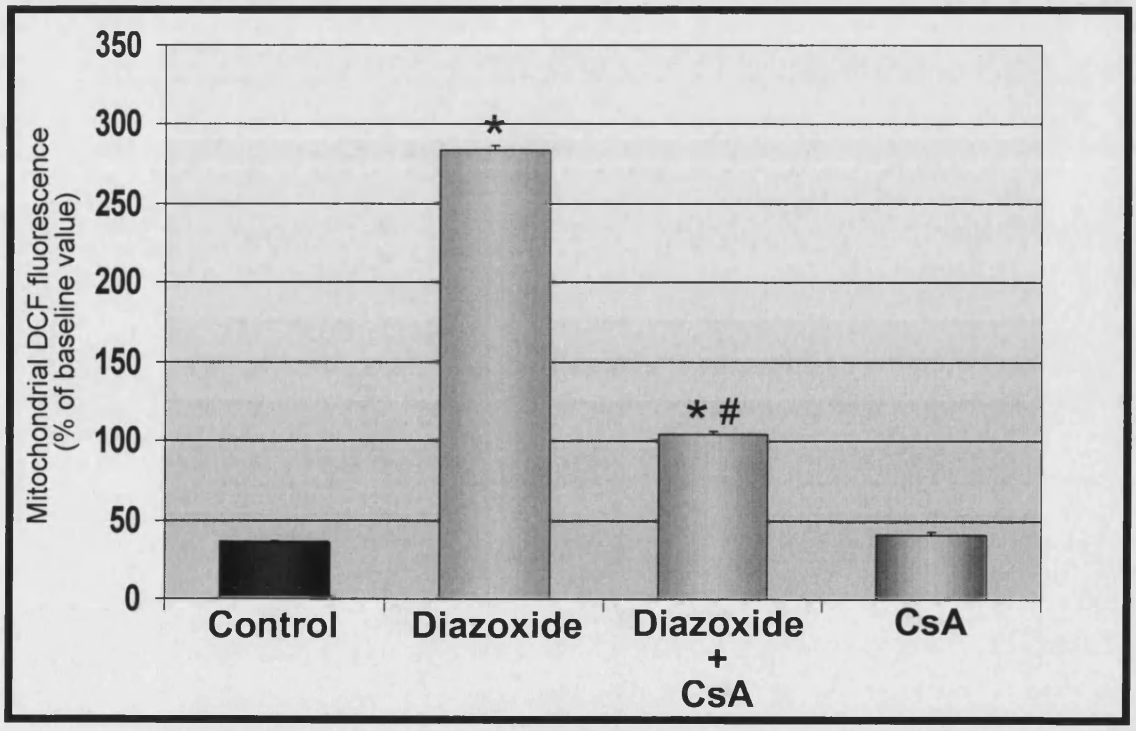
7.5.4.1 Exclusions

We used 2 male Sprague-Dawley rat hearts for mitochondrial isolation of which 0 were excluded (see section 3.3 for exclusion criteria).

7.5.4.2 Mitochondrial DCF Fluorescence Data

Arithmetic mean values of the median mitochondrial DCF fluorescence intensities were determined for each sample, and were expressed as a percentage of the baseline. The mitochondrial DCF fluorescence values in the control group increased over 30 minutes by 35.9±1.1%, whereas in the presence of diazoxide it increased by 283.9±3.1%. The diazoxide-induced increase in DCF fluorescence was abolished in the presence of the mPTP inhibitor, CsA, as the mitochondrial DCF fluorescence only increased by 104.0±1.7% over 30 minutes. In the presence of CsA alone, the mitochondrial DCF fluorescence increased by 40.3±2.2%.

Figure 7.7: Changes in Mitochondrial DCF Fluorescence. In control, the mitochondrial DCF fluorescence increased by 36% over the 30 minute period. Treatment with either diazoxide (30 μmol/l) induced an increase in mitochondrial DCF fluorescence, indicating the generation of reactive oxygen species (ROS). The presence of the mPTP inhibitor, cyclosporin-A (CsA) prevents the increases in mitochondrial DCF fluorescence, suggesting that the diazoxide-induced increase in ROS requires mPTP opening. CsA alone does not differ from control. Values are means of the median mitochondrial DCF fluorescence for that group ±SEM expressed as a % of the baseline fluorescence.*P<0.001 compared to control. .#P<0.001 compared to diazoxide treatment.



7.5.5 Discussion

In this part of the study we confirm the findings of previous studies demonstrating that diazoxide induces an increase in mitochondrial ROS.([72;116]) We show for the first time that the diazoxide-induced production of ROS requires mPTP opening since it was attenuated in the presence of the mPTP inhibitor, CsA. Importantly, CsA alone did not influence DCF fluorescence, suggesting that CsA does not exert a free radical scavenging effect. We postulate that it is transient (low-conductance) mPTP opening which is required to mediate the diazoxide-induced mitochondrial ROS release. The findings from this part of the study provide one possible mechanism by which transient (low-conductance) mPTP opening mediate the protection of diazoxide, in that it acts as a channel for mitochondrial ROS release. Therefore, in the infarct studies, we observed that the diazoxide-induced reduction in infarct size was abrogated in the presence of CsA. We can postulate that the protection was blocked because CsA prevented to release of mitochondrial ROS into the cytosol to activate the kinases required for protection. Further studies are required to determine whether mPTP opening is required to mediate the mitochondrial ROS release induced by IPC and mitochondrial uncoupling.

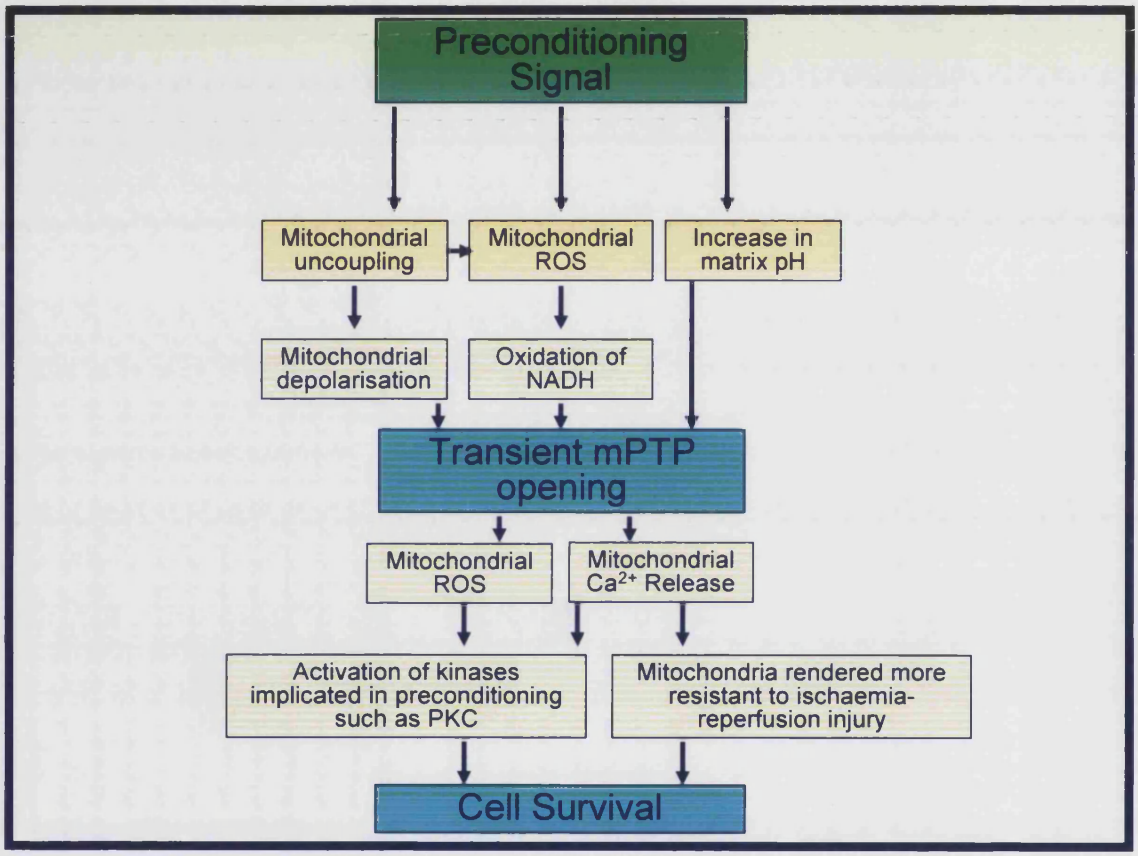
7.6 Discussion and Conclusion

In this section of the study, we demonstrated that transient (low-conductance) mPTP opening is induced during the preconditioning phase and is required for the protection associated with preconditioning and mitochondrial uncoupling. Our provisional data suggests that transient (low-conductance) mPTP opening acts as a channel for the release into the cytosol of mitochondrial ROS generated in response to the preconditioning stimulus.

The mechanism by which preconditioning induces transient (low-conductance) mPTP opening has not been examined in this study and forms the basis of further study. However, several mechanisms can be postulated (see figure 7.8). Preconditioning may induce transient (low-conductance) mPTP opening by: (1) inducing matrix alkanisation;([110;132]) (2) generating mitochondrial ROS([72;116]) which oxidises NADH; and/or (3) inducing mitochondrial uncoupling.([108;150;257;846]) All these effects would act to induce transient (low-conductance) mPTP opening, which can be induced by matrix alkanisation,([686]) the oxidation of NADH,([659]) and mitochondrial uncoupling.([906;907])

Other mechanisms by which transient (low-conductance) mPTP opening mediates preconditioning-induced protection include (see figure 7.8): (1) Reducing mitochondrial Ca^{2+} load, as demonstrated by Katoh and colleagues([266]) who demonstrated that diazoxide (albeit at a high concentration) induces mitochondrial Ca^{2+} efflux via mPTP opening in myocytes, and Holmuhamedov and colleagues,([257;258]) who demonstrated that diazoxide (albeit at a high concentration) induces mitochondrial Ca^{2+} efflux via mPTP opening in mitochondria; and (2) Facilitating Ca^{2+} signalling and subsequent kinase activation, although the evidence for this is not available, except for the fact that calcium preconditioning([372;375;378]) (in which a transitory increase in intracellular Ca^{2+}) triggers preconditioning-induced protection by activating PKC, may induce transient (low-conductance) mPTP opening.

Figure 7.8: Hypothetical Scheme Depicting the Role of Transient mPTP Opening in Preconditioning. The preconditioning signal induces transient (low conductance) mPTP opening by: (1) Uncoupling mitochondria which induces transient mPTP opening by mitochondrial membrane depolarisation or ROS release; (2) Generating ROS which induces transient mPTP opening by oxidising NADH; and (3) Increasing matrix pH. Transient (low conductance) mPTP opening protects the heart by inducing the release of mitochondrial ROS and Ca^{2+} which activate kinases implicated in preconditioning such as PKC. In addition, mitochondrial Ca^{2+} release renders the mitochondria more resistant to ischaemia-reperfusion injury.



The findings from this study add to the amounting evidence that the modulation of mitochondrial function is pivotal to preconditioning-induced protection. Interestingly, transient (low-conductance) mPTP opening may be considered the mitochondrial response to a brief amount of stress, such as that induced by a preconditioning stimulus. Transient (low-conductance) mPTP opening then triggers downstream mediators of preconditioning such as PKC by mediating mitochondrial ROS release.

Chapter Eight: SUMMARY AND DISCUSSION

Novel therapeutic approaches are required to improve upon the current treatment strategies employed for coronary artery disease, in order to reduce the mortality and morbidity from this common condition. Current advances in the areas of primary prevention (HMG-CoA reductase inhibitors, lifestyle changes, anti-platelet agents, ACE-inhibitors) and secondary prevention (anti-platelet agents, thrombolytic agents, primary PTCA, drug-eluting stents, and anti-thrombotic therapy) have made and will continue to make a significant impact on patient mortality and morbidity.

The phenomenon of myocardial preconditioning has the potential to deliver a powerful cardio-protective effect against ischaemia-reperfusion injury, but by definition, it necessitates intervening before the index ischaemic episode, which in the setting of an acute myocardial infarction, is beyond the control of the operator. Therefore, the role for agents which cardio-protect by preconditioning the heart, will be in targeting patient populations, that are either at high-risk of a major coronary occlusive event (such as patients presenting with an unstable coronary artery syndrome) or in those patients undergoing cardiac surgery.

An alternative cardio-protective strategy which intervenes, after the index ischaemic event as an adjunct to current reperfusion treatment strategies (whether that be by thrombolysis or primary PTCA), offers a powerful protective approach, and importantly it is under the control of the operator. The opening of the mitochondrial permeability transition pore (mPTP) during the first few minutes of reperfusion, that follows the index ischaemic period is a critical determinant of cell death, and therefore presents a potential target for cardio-protection.

This thesis examined the role of the mPTP as a target for cardio-protection against ischaemic-reperfusion injury. We found that inhibiting the mPTP opening which takes place at reperfusion is a common target for cardio-protection, irrespective of whether the protection was mediated by interventions applied solely at the time of reperfusion or by myocardial preconditioning. In addition, we presented evidence that, prior to the index ischaemic period, transient (low-conductance) opening of the mPTP may paradoxically, act as a mediator of preconditioning-induced protection.

The main findings of this thesis were: (1) The mPTP is a critical determinant of cell death in the setting of ischaemia-reperfusion injury, and pharmacologically inhibiting its opening, solely at the time of reperfusion, protects the heart against lethal reperfusion injury, as

evidenced by a reduction in infarct size; (2) Myocardial preconditioning induced by either ischaemia or pharmacological agents, protects the heart by inhibiting the opening of the mPTP, which occurs during the first few minutes of reperfusion; (3) Ischaemic preconditioning induces the activation of the pro-survival kinase cascades, PI3K-Akt and MEK1/2-Erk1/2, during the first few minutes of post-ischaemic-reperfusion, and these kinase cascades may protect the heart by inhibiting the mPTP opening; (4) Transient (low-conductance) opening of the mPTP during the preconditioning phase, may mediate the protection associated with ischaemic and pharmacological preconditioning and mitochondrial uncoupling, by acting as a channel for the mitochondrial release of reactive oxygen species (ROS), enabling the activation of kinases implicated in protection such as PKC.

The mPTP as a Mediator of Lethal Reperfusion Injury

In chapter 4, we demonstrated, using the isolated perfused rat heart model, that the presence of the pharmacological mPTP inhibitors, cyclosporin-A or sanglifehrin-A during the first few minutes of reperfusion, protected the heart against lethal reperfusion injury, as evidenced by a near 50% reduction in infarct size. Importantly, we demonstrated that targeting the first few minutes of reperfusion was critical to the protective effect of these mPTP inhibitors, which supports the experimental findings that the mPTP opens during the first few minutes of post-ischaemic reperfusion.([52;470;776;777]) The findings from this part of the study suggested that the opening of the mPTP during the first few minutes of reperfusion is a critical determinant of cell death, and that inhibiting its opening provide a potential target of cardio-protection.

The mPTP as a Target for Cardio-Protection in Myocardial Preconditioning

In chapter 5, we demonstrated, using several different methodological approaches, that the protection associated with the phenomenon of myocardial preconditioning was mediated by the inhibition of mPTP opening at the time of reperfusion, suggesting that the mPTP is a common target for cardio-protection, irrespective of whether the protection is mediated by interventions applied solely at the time of reperfusion or by myocardial preconditioning. Several studies have since been published, which confirm that inhibiting mPTP opening is critical to the protection associated with myocardial preconditioning.([803;846;859;881;882;892])

In the initial infarct studies, we demonstrated that pharmacologically opening the mPTP during the first few minutes could abrogate the protection induced by both ischaemic and

pharmacological preconditioning, suggesting that preconditioning protects by inhibiting the mPTP opening at the time of reperfusion. Halestrap's group have confirmed that IPC protects the heart by inhibiting the mPTP opening that occurs in the first few minutes of reperfusion.([803]) Using a similar approach, we demonstrated that the protection associated with delayed preconditioning could also be abrogated by pharmacologically opening the mPTP at the time of reperfusion. This finding has been confirmed in a study by Rajesh and colleagues using a similar methodological approach.([882]) Lemaster's group have subsequently demonstrated that heat shock (which protects by a delayed preconditioning effect) suppresses mPTP opening in isolated liver mitochondria.([892])

We demonstrated that the preconditioning mimetic, diazoxide could inhibit the mPTP opening induced by mitochondrial Ca^{2+} loading, a finding which was confirmed by Korge and colleagues.([846]) In addition they demonstrated that a PKC agonist had the same inhibitory effect on mPTP opening. Using a model for inducing and detecting mPTP opening in the intact cell, we demonstrated that hypoxic and pharmacological preconditioning protected myocytes against oxidative stress by inhibiting mPTP opening, a finding which has been confirmed in a recent study by Akao and colleagues.([859])

Preconditioning may inhibit mPTP opening at the time of reperfusion by several possible mechanisms including an indirect effect, in which known inducing factors of mPTP opening, such as mitochondrial Ca^{2+} load,([52;239;257;258;261;262]) ATP depletion, ([159;234;267]) and oxidative stress([77-79;278]) are counteracted by the effects of preconditioning, such that mPTP opening does not occur during the first few minutes of reperfusion. In this regard, we demonstrated that diazoxide appeared to attenuate the oxidation of NADH in the setting of oxidative-stress-induced mPTP opening. Additionally diazoxide appeared to depolarise mitochondrial membrane potential and induce flavo-protein oxidation albeit after a prolonged incubation, which may support the role for preconditioning in attenuating mitochondrial Ca^{2+} load.

Alternatively, a component of preconditioning may target and inhibit mPTP opening directly. In this regard, Baines and colleagues demonstrated that PKC-ε, may interact with and inhibit mPTP opening.([881]) Preconditioning may also inhibit mPTP opening by modulating the ratio of pro- and anti-apoptotic proteins of the Bcl-2 family. ([243;244;882;894]) In chapter 6, we examined the role of the pro-survival kinases as a potential candidate for mediating the inhibition of mPTP opening, as the mechanism for preconditioning-induced protection.

The Pro-Survival Kinases as a Target for Cardio-Protection in Myocardial Preconditioning

In chapter 6, we examined the role of the pro-survival kinases PI3K-Akt and MEK1/2-Erk1/2, *at the time of reperfusion,* as a mediator of preconditioning protection. Previous studies had demonstrated that the activation of these kinase cascades prior to the index ischaemic episode was required for protection as a preconditioning trigger.([80-82]) We demonstrated that IPC protects the heart by activating these kinases at the time of reperfusion, and we provided preliminary data that these kinase cascades may protect by inhibiting mPTP opening. In the next part of the thesis, we examined the role of the mPTP as a potential mediator of preconditioning-induced protection.

The mPTP as a Mediator for Cardio-Protection in Myocardial Preconditioning

In chapter 7, we examined the role of transient (low-conductance) mPTP opening, which does not lead to cell death and may be beneficial to the cell, as a potential mediator of preconditioning-induced protection. Previous studies have suggested that this form of mPTP opening shares some features with preconditioning. For example, (1) Both preconditioning([52;239;257;258;261;262]) and transient (low-conductance) mPTP opening([266;674;835]) have been demonstrated to attenuate mitochondrial Ca^{2+} loading; (2) Both preconditioning.([49;67;72]) and transient (low-conductance) mPTP opening,([840]) may induce the release of mitochondrial ROS (3) Mitochondrial uncoupling is a critical process in both preconditioning([150;236;237]) and transient (low-conductance) mPTP opening.([907]) Using the isolated perfused rat heart we demonstrated that pharmacologically inhibiting mPTP opening during the preconditioning phase abrogated the protection associated with both ischaemic and pharmacological preconditioning. Using a myocyte model for detecting transient mPTP opening, we demonstrated that diazoxide induces transient (low-conductance) mPTP opening, confirming the findings of Katoh and colleagues.([266]) Finally, using isolated mitochondria, we demonstrated that the diazoxide-induced generation of mitochondrial ROS requires mPTP opening. Therefore, the findings from this part of the study suggest that transient (low-conductance) opening of the mPTP mediates both preconditioning and mitochondrial uncoupling-induced protection by acting as a channel for mitochondrial ROS release. Evidence in the literature suggest that diazoxide can induce mPTP opening and promote mitochondrial Ca^{2+} efflux.([257;258;266])

Given that we have demonstrated that transient (low-conductance) opening of the mPTP prior to the index ischaemic episode mediates preconditioning-induced protection, and

the recent suggestion that the adenine nucleotide translocase, which is believed to be a component of both the mPTP,([909]) may also form part of the mitochondrial K_{ATP} channel,([146]) it is intriguing to speculate on the possibility that the mPTP may be masquerading as the mitochondrial K_{ATP} channel, such that preconditioning-induced protection is mediated by transient (low-conductance) opening of the mPTP rather than opening of the mitochondrial K_{ATP} channel.

A Dichotomous Role for the mPTP in Myocardial Protection

Interestingly, the findings from this thesis which suggest that the mPTP may mediate preconditioning-induced protection implicate a dichotomous role for mPTP opening in myocardial preconditioning. On the one hand the prolonged high-conductance mPTP opening which occurs during the first few minutes of reperfusion mediates cell death and is inhibited by preconditioning, whereas on the other hand, the transient (low-conductance) form of mPTP opening which occurs physiologically is activated prior to the index ischaemic period and may mediate preconditioning-induced protection by acting as a channel for mitochondrial ROS release. Combining these two effects of preconditioning, one can conclude that preconditioning protects the heart by inducing the transient (low-conductance) mPTP opening during the preconditioning phase, which sets into motion mechanisms which protect the mitochondria against the insult of ischaemia-reperfusion injury, such that at the time of reperfusion, the prolonged high-conductance mPTP opening is suppressed.

Interestingly, in the context of myocardial preconditioning other factors have been demonstrated to play a dichotomous role. For example, ROS have been implicated as trigger/mediators of preconditioning-induced protection when released in small amounts during the preconditioning phase.([49;67]) However, at the time of post-ischaemic reperfusion, ROS are a major determinant of lethal reperfusion injury and studies have demonstrated that preconditioning can attenuate the production of ROS at the time of reperfusion.([78;79]) These examples of dichotomy in cardio-protection echo the phenomenon of ischaemic preconditioning as originally described by Murry and colleagues in 1986,([5]) in which a brief episode of ischaemia and reperfusion, paradoxically protected the heart against a subsequent lethal episode of ischaemia. Therefore, whether it be a brief episode of ischaemia, a small burst of ROS or transient (low-conductance) opening of the mPTP opening, these examples of cellular stress are able to trigger a cellular innate adaptive response to further episode of cellular stress.

Chapter Nine: CONCLUSION

9.1 Summary of Findings

This thesis has examined the role of the mitochondrial permeability transition pore (mPTP) in myocardial protection. We have demonstrated that the opening of the mPTP during the first few minutes of reperfusion is a critical determinant of cell death in the setting of ischaemia-reperfusion injury, and therefore inhibiting its opening by either myocardial preconditioning or the administration of agents solely at the time of reperfusion, is an effective cardio-protective strategy. Importantly we demonstrated that the mPTP appears to be a common target of myocardial protection, irrespective of whether the protection was mediated by interventions applied solely at the time of reperfusion or by myocardial preconditioning. In addition, we present evidence that, prior to the index ischaemic period, the transient (low-conductance) opening of the mPTP may paradoxically, act as a mediator of preconditioning-induced protection, by acting as a mitochondrial channel for ROS release. The findings from this study suggest a dual role for the mPTP in the setting of myocardial protection, and importantly, suggest that myocardial preconditioning can protect the heart by modifying crucial events which take place during the first few minutes of reperfusion.

9.2 Clinical Implications

A significant finding from this study, is that the powerful cardio-protective phenomenon that is myocardial preconditioning appears to protect the heart by modifying events which occurs during the first few minutes of post-ischaemic reperfusion. In this thesis, we have provided evidence that preconditioning protects the heart by inhibiting the mPTP opening which occurs during the first few minutes of reperfusion. In addition, preconditioning activates cellular innate pro-survival mechanisms, such as the PI3K-Akt and MEK1/2-Erk1/2 kinase cascades, during the first few minutes of reperfusion. Therefore, the first few minutes of reperfusion offer a potential target for cardio-protection which would be under the control of the operator and from the findings of this thesis, targeting the mPTP opening which occurs at the time of reperfusion, can offer protection that is comparable to myocardial preconditioning.

Targeting mPTP Opening at the Time of Reperfusion

Importantly, we demonstrated that the opening of the mPTP during the first few minutes of reperfusion was a major determinant of lethal reperfusion injury and was responsible for up to 50% of the infarct size sustained following an episode of lethal ischaemia. We demonstrated that inhibiting mPTP opening during the first few minutes of reperfusion, by either administering pharmacological mPTP inhibitors or by preconditioning the myocardium produced significant protection.

Suitable pharmacological agents need to be developed which specifically inhibit the mPTP opening, that are safe and can achieve therapeutic levels rapidly, for use at the time of reperfusion, in the clinical settings of ischaemia-reperfusion injury, such as at the time of reperfusion (by either thrombolysis or primary PTCA) following an acute myocardial infarction and at the time of cardiac surgery. However, the drug would have to be administered as an adjunct to the reperfusion treatment strategy to be effective, as we have demonstrated in this thesis, that targeting the first few minutes of post-ischaemic reperfusion is critical to protection. Pharmacological agents we have used in this thesis to inhibit mPTP opening such as cyclosporin-A, which is used clinically as an immunosuppressant following transplant surgery, can produce serious side effects, and in addition the use of this agent requires 2-3 days to achieve steady state serum levels. Therefore, the local intra-coronary delivery of a small concentration of CsA, at the time of primary angioplasty for an AMI, may provide a potential approach for harnessing the protection gained from inhibiting mPTP opening at the time of reperfusion. An alternative approach would be to administer pharmacological agents, which are safe for clinical use, and have been demonstrated to activate the cells own pro-survival pathways.

Targeting the Pro-Survival Kinase Pathway at the Time of Reperfusion

The findings from this thesis suggest that ischaemic preconditioning protects the heart by activating the pro-survival kinase pathways, PI3K-Akt and MEK1/2-Erk1/2 during the first few minutes of reperfusion. We also provided preliminary data suggesting that these kinase cascades may protect at the time of reperfusion by inhibiting mPTP opening. Therefore, the cardio-protective benefits of inhibiting mPTP opening during the first few minutes of reperfusion may be achieved by administering pharmacological agents which activate these pro-survival kinase pathways, termed the Reperfusion Injury Salvage Kinase (RISK)-Pathway. Suitable

pharmacological agents, which have been demonstrated to activate these kinase cascades, that could be administered during the first few minutes of reperfusion, as an adjunct to current reperfusion therapy are, HMG-CoA reductase inhibitors,([522]) ACE-inhibitors,([524]) insulin([530]) or several other growth factors.([519]) These agents may provide a potential approach to salvaging viable myocardium and limiting infarct size in patients presenting with an acute myocardial infarction.

Despite clinical studies reporting benefit from the early administration of HMG-CoA reductase inhibitors following an acute myocardial infarction,([910;911]) as yet no study has been undertaken which examines whether these drugs offer cardio-protection when given during the first few minutes of reperfusion following an acute myocardial infarction, as an adjunct to thrombolysis or primary PTCA.

It would be interesting to speculate whether the benefits reported in the clinical trials (such as the DIGAMI([620]) and ECLA([621]) and other trials([622])), reporting benefits from the treatment of glucose, insulin and potassium (GIK) therapy at the time of reperfusion, following an acute myocardial infarction, were due to inhibition of mPTP opening. Already, a large randomised control clinical study (named GIK II) is underway, examining the benefits of glucose insulin therapy (GIK) given at time of reperfusion in patients presenting with an acute myocardial infarction.([626])

Recent studies have shown that fluoroscopic-guided intramyocardial injection in the pig model is a feasible and safe procedure for targeting the delivery of therapeutic agents to the area of myocardium at risk from ischaemia-reperfusion injury.([912]) Therefore the local delivery of growth factors themselves or the adenoviral vectors (carrying mutated genes which over-express growth factors) may provide a potential method for targeting and up-regulating the RISK-pathway in the clinical setting of reperfusion. Alternatively for patients undergoing an anticipated episode of ischaemia-reperfusion injury, such as during CABG surgery or elective coronary angioplasty, gene transfer may be a possible method of delivering growth factors to myocardium at risk of lethal reperfusion-induced injury.

9.3 Future Directions

The mPTP as a Mediator of Lethal Reperfusion Injury

We have demonstrated that the opening of the mPTP during the first few minutes of reperfusion is a critical determinant of cell death and that inhibiting its opening offers a powerful target for myocardial protection. Further experimental studies are required to investigate newer classes of drugs which inhibit mPTP opening and which can be demonstrated to offer protection against lethal reperfusion injury by inhibiting mPTP opening. In this regard, the new class of mPTP inhibitors,([682]) which appear to interact and inhibit mPTP opening by targeting the VDAC, a presumed outer membrane component of the mPTP, may provide a potential approach.

Furthermore, it would be interesting to determine whether other interventions which have been demonstrated to protect the heart against lethal reperfusion injury, when applied solely at reperfusion, such as the phenomenon of 'ischaemic post-conditioning' or the opening of the mitochondrial K_{ATP} channel, do so by inhibiting mPTP opening.

The mPTP as a Target for Cardio-Protection in Myocardial Preconditioning

In this thesis, we have demonstrated that inhibiting mPTP opening at the time of reperfusion, is critical to the protection induced by both ischaemic and pharmacological preconditioning. Further studies are required to elucidate the mechanisms that mediate the inhibition of mPTP opening, especially in light of previous studies which suggest that certain components of preconditioning such as PKC can interact with and inhibit mPTP opening directly.([881]) However, most evidence would support the proposition that the inhibition of mPTP opening is due to the beneficial effects on mitochondrial function (such as mitochondrial Ca^{2+} load, mitochondrial energy production, and reduced production of oxidative stress) which are induced by a preconditioning stimulus, and these factors then act in concert to reduce the opening probability of the mPTP at the time of reperfusion.

The Pro-Survival Kinases as a Target for Cardio-Protection in Myocardial Preconditioning

It would be both important to demonstrate that agents which protect at reperfusion by activating the pro-survival kinases (such as insulin,([530]) HMG-CoA reductase inhibitors([522]) and ACE-inhibitors([524])), PI3K-Akt and MEK1/2-Erk1/2 actually protect the heart against lethal reperfusion

injury by inhibiting the mPTP opening during the first few minutes of reperfusion. Our preliminary data (chapter 6) demonstrated that insulin (which activates the PI3K-Akt kinase cascades) can protect myocytes against oxidative stress (a major determinant of lethal reperfusion injury), by inhibiting the mPTP opening.

The mPTP as a Mediator for Cardio-Protection in Myocardial Preconditioning

The findings that the mPTP can act as a mediator of preconditioning-induced protection prior to the index ischaemic episode (chapter 7), underscores the importance of modulating mitochondrial function in preconditioning-induced protection. Further work is required to determine the contribution of transient (low-conductance) mPTP opening to preconditioning-induced protection, and to confirm the role of this form of mPTP opening in mediating the mitochondrial release of ROS, in response to a preconditioning stimulus.

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